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Molecular control of neurogenesis in the regenerating central nervous system of the adult zebrafish

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Abstract

In contrast to mammals, adult zebrafish display cellular regeneration of lost motor neurons and achieve functional recovery following a complete spinal cord transection. Using adult zebrafish as a model to study how key developmental pathways can be re-activated to regulate neuroregeneration in cellular recovery, I addressed the following questions:

1) What is the role of Notch signalling during regenerative mechanisms in the lesioned spinal cord of the adult zebrafish? 2) What is the role of Notch overexpression in neurogenesis in the adult zebrafish retina?

3) Which additional signalling pathways are involved in the generation of motor neurons during spinal cord regeneration in adult zebrafish?

1) In the main part of my thesis I have investigated the role of Notch signalling during spinal cord regeneration. The Notch pathway has been shown to regulate neural progenitor maintenance and inhibit neuronal differentiation in the vertebrate nervous system. In the injured mammalian spinal cord, increased Notch signalling is held partly responsible for the low regenerative potential of endogenous progenitors to generate new neurons. However, this is difficult to test in an essentially non-regenerating system. We show that in adult zebrafish, which exhibit lesion-induced neurogenesis, e.g. of motor neurons from endogenous spinal progenitor cells, the Notch pathway is also reactivated. I over-activated the Notch pathway by forced expression of a heat-shock inducible active domain of notch in spinal progenitor cells. I observed that although apparently compatible with functional regeneration in zebrafish, forced activity of the pathway

significantly decreased progenitor proliferation and motor neuron generation. Conversely, pharmacological inhibition of the pathway increased proliferation and motor neuron numbers. Thus in summary our work demonstrates that Notch is a negative signal for regenerative neurogenesis in the spinal cord.

Importantly, we show for the first time that spinal motor neuron regeneration can be augmented in an adult vertebrate by inhibiting Notch signalling.

2) While in the lesioned spinal cord, over-activation of Notch attenuated neurogenesis, I observed that in the unlesioned retina the same manipulation led to strong proliferation of cells in the inner nuclear layer, presumable Müller glia cells which are the retinal progenitor cells. This coincided with an increase in eye size in adult zebrafish. These preliminary findings provide the first hint that the role of Notch may differ for different adult progenitor cell pools and will lead to future investigations of Notch induced neurogenesis in the retina.

3) We have evidence from previous studies that the dopamine and retinoic acid (RA) signalling pathways may be involved in the generation of motor neurons in the adult lesioned spinal cord. Using in situ hybridisation, I assessed the gene expression patterns a) for all D2-like receptors and b) candidate genes that relate to the RA pathway in the adult lesioned spinal cord to identify the signalling components.

a) I found that only the D4a receptor was upregulated in spinal progenitor cells in the ventricular zone rostral to the lesion site, but not caudal to it. This correlates with other results showing that dopamine agonists increase motor neuron regeneration rostral, but not caudal to a spinal lesion site.

b) I observed a strong increase in the expression of Cyp26a, a RA catabolising enzyme, in the ventricular progenitor zone caudal to the lesion site, in contrast to the weak expression rostrally. Crabp2a, a cellular retinoic acid binding protein, was also upregulated rostral and in close proximity to the lesion site in a subpopulation

of neurons located ventrolaterally in the spinal cord.

In summary, we show that the Notch pathway negatively regulates neurogenesis in the spinal cord in contrast to the retina and provide evidence that dopamine from the brain signals via the D4a receptor to promote the generation of motor neurons in addition to RA, which may also play a role in this process. These insights into adult neural progenitor cell activation in zebrafish may ultimately inform therapeutic strategies for spinal cord injury and neurodegenerative diseases such as motor neuron disease.

Statement of original contribution

The work in this thesis has been performed by the candidate, Tatyana Dias, unless specifically stated otherwise.

Tatyana Dias

10/01/2012

Dedication

To Veruschka my baby sister,

This is for you

Acknowledgements

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1. General introduction

1.1 Damage to the spinal cord and barriers to successful regeneration in mammals

The pathological effects of spinal cord injury are complex and result in myelopathy, inflammation, formation of a glial scar, damage to the white matter and myelinated fibres that carry sensory information to and from the motor neurons to the brain. This is associated with a huge loss of neurons and glia (Lindvall and Kokaia, 2010; Ronaghi et al., 2010). Neurodegenerative diseases such as amyotrophic lateral sclerosis or motor neuron disease, a devastatingly fatal adult-onset condition, are characterized by the loss of motor neurons in the anterior horns of the spinal cord (Talbot and Ansorge, 2006).

De novo neuronal production in the mammalian spinal cord is highly inefficient and functional deficits are permanent. There is little or no neurogenesis in the uninjured mammalian spinal cord. Mammalian ependymal spinal cord progenitors with radial glia features respond to an injury, however they ultimately become glial cells and contribute to the glial scar (Meletis et al., 2008; Barnabé-Heider et al., 2010). Interestingly, these spinal progenitors have the intrinsic capacity to generate neurons when placed in a suitable environment (Shihabuddin et al., 2000). Activation of Notch signalling has been held responsible for the low regenerative potential of spinal progenitors to generate neurons in mammals. This suggests that the environment plays an important role in the initiation of repair mechanisms. Furthermore, attenuation of Notch signalling increased neuronal differentiation from spinal progenitors (Yamamoto et al., 2001).

Several molecules have been implicated in contributing to the inhibitory environment and a limited regenerative potential in the spinal cord. Myelin proteins such as Nogo-A (Oertle et al., 2003), oligodendrocyte myelin glycoprotein (OMgp) (Wang et al., 2002) and myelin associated glycoprotein (MAG) (Mukhopadhyay et al., 1994) are known to inhibit neurite outgrowth and axonal

regeneration in the mammalian CNS. In addition, chondroitin sulfates in the glialscar and secreted axon-repellent (class 3) semaphorins also inhibit axonal regeneration (Niclou et al., 2003; Silver and Miller, 2004; Niclou et al., 2006). Thus, axonal regrowth and neurogenesis in the mammalian spinal cord is impaired. It is therefore pivotal to elucidate molecular mechanisms by which we can promote regeneration in the mammalian CNS. The therapeutic potential of endogenous progenitor/stem cell manipulations offers the possibility for research into adult neurogenesis, the promise of drug discovery and/or informed neuronal replacement therapy for spinal cord injury and neurodegenerative diseases such as motor neuron disease (Roskams and Tetzlaff, 2005; Bareyre, 2008). The target cell type for designing therapeutic strategies are the motor neurons.

1.2 Specification of motor neurons

Shh secreted from the floor plate and the notochord is required for the generation of motor neurons (Patten and Placzek, 2000). The specification of motor neurons takes places in three sequential steps. In the first step towards specification, the ventral progenitor domains are defined by the expression of homeodomain (HD) transcription factors (Briscoe and Ericson, 2001). Class I HD proteins (Pax7, Pax6, Dbx1/2 and Irx3) are repressed by shh signalling whereas the class II HD proteins (Nkx6 and Nkx2) require shh for their expression. The cross-repressive interactions between the class I and class II HD proteins sharpen and define the ventral progenitor domains into five types p0, p1, p2, pMN and p3 (Lee and Pfaff, 2001). In the second phase of specification, progenitors express domain-restricted determinants of neuronal subtype identity (Muhr et al., 2001). In particular, motor neuron progenitors express class I (Pax6 and not Irx3) and class II (Nkx6 and not Nkx2) HD proteins.

The motor neuron progenitors also express the basic-helix-loop-helix (bHLH) transcription factor Olig2 (Mizuguchi et al., 2001; Novitch et al., 2001). In the final phase, before the first postmitotic neurons are born, HD proteins interact with

bHLH transcription factors to initiate specific programs of differentiation (Muhr et al., 2001). The progenitors in the pMN domain give rise to motor neurons and oligodendrocytes (Kimmel et al., 1994). Nkx6 is activated by shh signalling which in turn induces the expression Olig2 in the pMN domain (Park et al., 2002). Olig2 serves as a determinant and promotes the expression of neurogenin2 (Ngn2) while repressing the expression of HD regulators like HB9, Lim3 and Islet1/2 (Novitch et al., 2001; Scardigli et al., 2001).

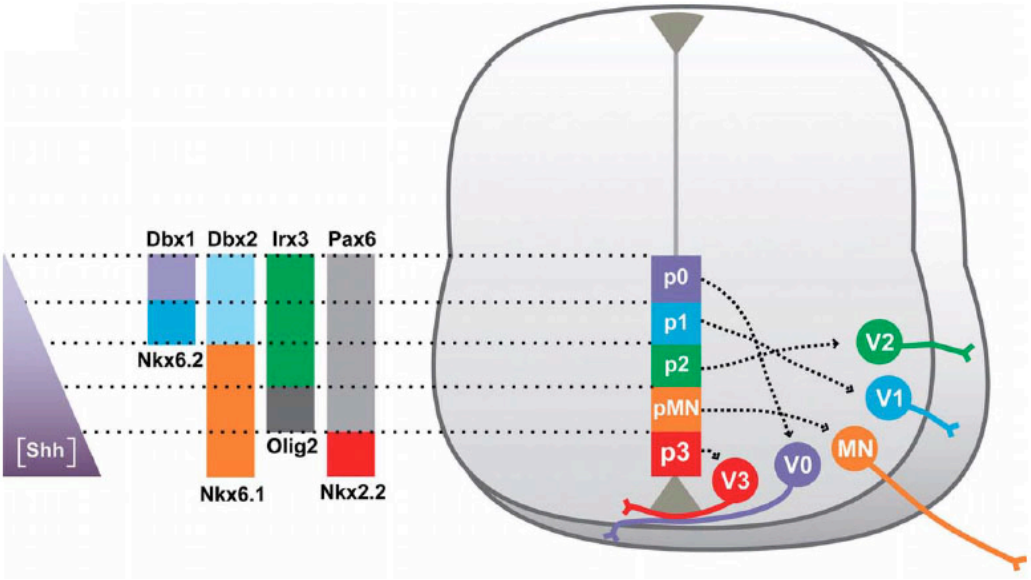

Fig. 1.1 Schematic diagram of the development of ventral spinal cord neurons in the neural tube. A ventro-dorsal gradient of shh and a combination of different class I and class II HD proteins delineate five different progenitor domains namely p0, p1, p2, pMN and p3 in the ventral neural tube. The progenitors in the p0, p1, p2 and p3 domains generate V0, V1, V2 and V3 interneurons respectively. pMN progenitors sequentially generate motor neurons and oligodendrocytes. Image taken from (Kullander, Vallstedt, 2007 ; Becker and Becker, 2007).

1.3 Zebrafish as a model organism for successful spinal cord regeneration

Zebrafish (*Danio rerio*) (Hamilton, 1822) are tropical freshwater fish that belong to the minnow family (Cyprinidae). Zebrafish serve as an excellent model to study neuronal development because the embryos are optically transparent and enable the

experimenter to follow individual cell movements and differentiation using time-lapse imaging. Furthermore, gene function can be studied using gain and loss of function experiments using RNA microinjection and anti-sense morpholinos (Eisen and Smith, 2008). Access to large variety of transgenic and mutant zebrafish allow for a wide selection of experimental tools. In addition, the ninth assembly of the zebrafish genome (Zv9) was published on Aug 2010

(http://www.ensembl.org/Danio_rerio/Info/Index). In contrast to mammals, adult zebrafish can regrow axons over the lesion site and functionally recover following a complete spinal cord transection (Becker et al., 1997; van Raamsdonk et al., 1998; Becker et al., 2004). Adult zebrafish can also replace damaged motor neurons from Olig2:GFP+ expressing ependymo-radial glial progenitors in the ventricular zone of the spinal cord (Reimer et al., 2008; Reimer et al., 2009). Regeneration in the zebrafish spinal cord is not a mere recapitulation of development but it is a highly ordered and regulated process. Spinal cord transection severs descending tracts originating from 20 different brain nuclei, ascending tracts to the brain, ascending intraspinal and descending axons (Becker et al., 1997; Becker et al., 2005). However, during spinal cord regeneration only a subset of neurons with a descending axon regrow their severed axon while none of the intraspinal and dorsal root ganglion neurons regenerate their ascending axon. During heart regeneration, transcription factors different to those expressed during heart development are expressed (Raya et al., 2003).

Given that zebrafish can replace damaged neurons and regrow axons over the lesion site, it would be worthwhile to study the molecular mechanisms during successful spinal cord regeneration. The information we gain from using this regenerating system would help us identify molecules or signalling pathways that contribute to the low regenerative potential in the mammalian spinal cord. As the signals for spinal cord development are highly conserved among vertebrates, identifying the mechanisms for lesion-induced neuronal replacement from

endogenous spinal progenitor cells in adult zebrafish may help inform subsequent therapy in mammals. Previous work has shown that shh signalling is required for the generation of motor neurons in the lesioned spinal cord. RA pathway genes were also upregulated following a spinal cord transection and may be involved in motor neuron generation (Reimer et al., 2009). The low regenerative potential of mammalian progenitors to generate functional neurons after a spinal injury has been attributed in part to the involvement of the Notch signalling pathway (Yamamoto et al., 2001).

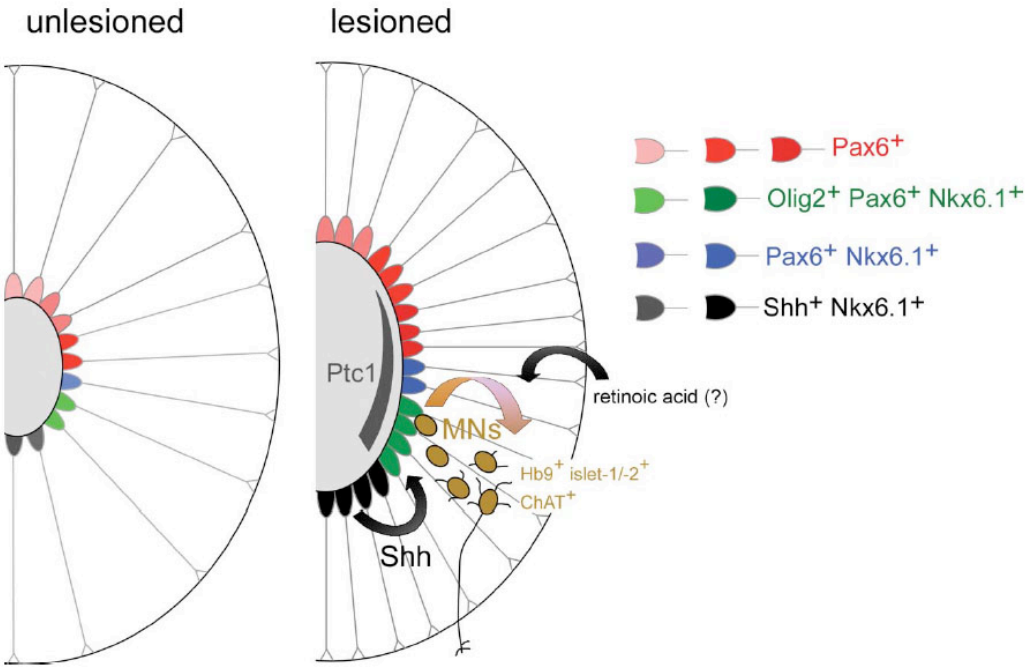

Fig 1.2 Schematic diagram of cross-sections of the unlesioned and lesioned spinal cord showing the events during motor neuron regeneration in the adult zebrafish. In the unlesioned spinal cord, combinations of different transcription factors were found in the same dorsoventral positions as in the embryonic neural tube. After a lesion, the expression of these transcription factors is enhanced and shh secreted from the floor plate influences the generation of newborn differentiating neurons that are HB9+/Islet1/2+. Some of these neurons later mature into ChAT+ motor neurons and are re-integrated into the spinal circuitry. RA genes are upregulated after a lesion by PCR (see chapter 1) speculating that RA may play a role in motor neuron generation. Image modified from (Reimer et al., 2009).

1.4 Retinoic acid (RA) pathway

Retinoic acid (RA) is a signalling molecule that has diverse roles in the development and regional specifications of embryonic tissues. In the CNS, it is essential during neural development for the patterning of the CNS, neural differentiation, axon outgrowth and the generation of specific neuronal subtypes. RA is a metabolite that is generated when vitamin A (retinol) is metabolized. Animals are incapable of synthesizing vitamin A so they extract it from their diet. Plants contain carotenoids and animal products have retinyl esters (retinoids). These dietary components are stored as retinoids in the liver, lungs, bone marrow and kidneys (Blomhoff and Blomhoff, 2006). The retinoids are transported from the hepatic and extrahepatic tissues to the cells that require them by retinol that is released in the bloodstream. Retinol circulates in the blood bound to the plasma retinol-binding protein 4 (RBP4) and is taken up by target cells through an interaction with a STRA6, a membrane bound receptor for RBP4 (Kawaguchi et al., 2007). On entering the cytoplasm it binds to cellular retinol-binding protein 1 (RBP1) and via a two-step process it is metabolized to all-trans RA. The first step is the mediated by retinol dehydrogenase 10 (RDH10) which converts retinol into a retinaldehyde (Ral) (Sandell et al., 2007). The retinaldehyde is then converted to all-trans RA by retinaldehyde dehydrogenases (RALDHs). The resulting RA can be released from the target cell and can be taken up by neighbouring cells (paracrine signalling) or it can act on its own nucleus (autocrine signalling). Cellular retinoic acid binding proteins (CRABPs) 1 and 2 assist the transport of RA from the cytoplasm of the cell into its nucleus (Budhu and Noy, 2002). In the nucleus, RA binds to a heterodimerized RA receptors (RARs) and retinoid X receptors (RXRs). There are three RAR genes (RARA, RARB and RARG) and three RXR genes (RXRA, RXRB and RXRG) and together they bind to the retinoic acid-response element (RARE) in the DNA. This interaction activates the transcription of target genes and the all-trans RA exits the nucleus. The cyp26 class of P450 enzymes then catabolizes the all-trans RA in the cytoplasm (see Fig 1.3)

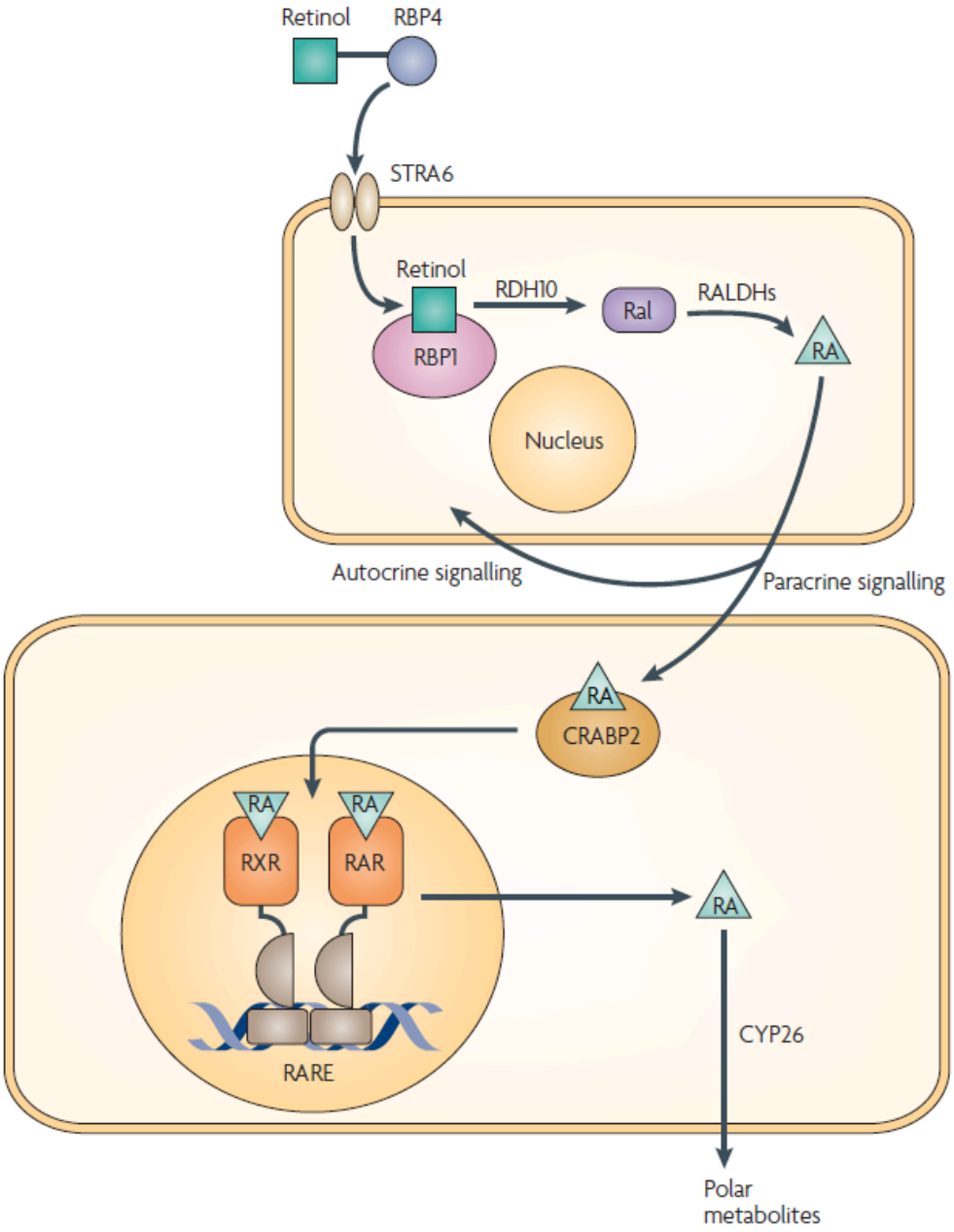

Fig 1.3. Schematic of the RA pathway. RBP4 = retinol-binding protein 4 plasma, RBP1 = retinol-binding protein 1, cellular, RDH10 = retinol dehydrogenase 10, RALDH = retinaldehyde dehydrogenases, RA = all-trans retinoic acid, CRABP2 = cellular retinoic-acid-binding protein 2, RXR = retinoic X receptor, RAR = RA receptor, RARE = retinoic acid-response element and CYP26 = Cytochrome P450 26. Image from (Maden, 2007).

1.4.1 RA signalling in the developing nervous system

In the embryonic nervous system, RA is essential for patterning and neuronal differentiation. RA contributes to patterning the antero-posterior and the dorso-ventral axes of the neural plate and neural tube. During the course of patterning in the antero-posterior axis, RA acts in concert with Wnts and fibroblast growth factors (FGFs) to specify the posterior hindbrain and the anterior spinal cord (Liu et al., 2001; Maden, 2002; Melton et al., 2004). In quail embryos that are deficient in RA, the posterior hindbrain fails to develop, thus suggesting a role for RA in its development (Maden et al., 1996). The cells in the posterior mesoderm synthesize RA, while the cells in the anterior mesoderm express the RA catabolising enzyme CYP26C1. These opposing signals create a gradient of RA that is able to pattern the neural plate to form the posterior hindbrain (Reijntjes et al., 2004; Glover et al., 2006).

In the developing chick spinal cord, RA can specify the p1 and p0 domains of the dorsal most part of the ventral neural tube along the dorso-ventral axis. The enzyme that generates RA, raldh2 is expressed in the somitic (paraxial) mesoderm which can induce the generation of V0 and V1 interneurons when cultured together with spinal cord explants (Pierani et al., 1999). The number of islet-1 positive motor neurons in the spinal cord was reduced in the absence of RA and neurites failed to extend into the periphery (Maden et al., 1996). Furthermore, RA has also been shown to be necessary for the specification of motor neuron subtypes. When brachial somites are placed at the thoracic level in the spinal cord, the motor neurons generated change from thoracic to the brachial type (Ensini et al., 1998). Later on development, brachial and lumbar motor neurons express raldh2 as a replacement for the paraxial mesoderm. Expression of raldh2 at the thoracic levels induces ectopic lateral motor column neurons (LMCs) (Sockanathan and Jessell, 1998). In contrast, if raldh2 expression in motor neurons is eliminated, the number of lateral and medial LMCs is reduced (Sockanathan et al., 2003; Vermot et al., 2005; Ji et al., 2006). RA from the paraxial somitic mesoderm contributes to the

specification of the lateral LMCs, whereas the neuronal source of RA is essential for the maintenance of the medial and lateral LMC populations (Maden, 2007).

1.4.2 RA signalling in the adult CNS

The levels of RA activity were increased due to increased levels of raldh2 after spinal cord injury in mice. The expression peaked 4-7 days after the damage was inflicted (Mey et al., 2005; Kern et al., 2007). In the uninjured spinal cord, meningeal cells and oligodendrocytes express Raldh2. After spinal cord injury it is expressed by meningeal cells that invade the damaged site (Maden, 2007). Spinal cord injury in mammals does not induce axonal regeneration and it could be because RARβ2 is not induced (Corcoran et al., 2002). Interestingly, transfection of RARβ2 in adult rat cortical neurons enabled them to grow longer and extend neurites, thus overcoming the inhibitory environment (Wong et al., 2006; Yip et al., 2006). During fin regeneration in the adult zebrafish, exogenous RA respecifies pattern in the caudal fin (White et al., 1994).

1.5 Role of Notch signalling during spinal cord regeneration

As mentioned above, the Notch pathway has been implicated as one of the causal factors contributing to the low regenerative potential in the mammalian spinal cord (Yamamoto et al., 2001). Since, zebrafish have this amazing capacity to regenerate their damaged spinal cord, in chapter 3 of this thesis I set out to investigate the role of Notch signalling during this reparative process.

The Notch pathway is regulated by a cell-cell communication process. The pathway initiates when a progenitor that responds to a neurogenic signal differentiates early and transiently expresses the delta/jagged ligands. On receptor stimulation, the Notch pathway is activated in the neighbouring cells where the γ-Secretase complex cleaves the Notch receptor. The activated form of Notch, i.e. the Notch intracellular domain (nicd), is released from the plasma membrane and translocates to the nucleus, where it forms an activator complex with the DNA-

binding protein suppressor of hairless (SuH) together with the co-activator mastermind (MAM). This activator complex then promotes the transcription of downstream targets of the her/hes (hairy and Enhancer of split E (SPL) related) family of genes. These genes are bHLH transcriptional repressors that antagonize proneural genes such as ash1a and the neurogenins, thus blocking early neuronal gene expression and inhibiting neuronal differentiation in the neighbouring cell (Yoon and Gaiano, 2005). In this way, Notch maintains the proliferative progenitor pool and enables the inhibited cells to adopt a differentiated state at a later stage (Imayoshi and Kageyama, 2011). In the zebrafish, there are four Notch receptors, namely Notch1a, Notch1b, Notch2 and Notch3. There are five delta ligands, namely deltaA, deltaB, deltaC, deltaD and Dll-4 (Haddon et al., 1998; Leslie et al., 2007). In addition, there are three jagged ligands, namely j1a, j1b and j2 (Figure 1.4) (Yeo and Chitnis, 2007).

Notch signalling is used iteratively and has diverse roles during the development of the central nervous system. It has been shown to be involved in the specification of neural fate, binary cell fate decisions and an instructive role in promoting gliogenesis. Furthermore, recent data indicates that Notch is required for neuronal function during adult neurogenesis (Fig 1.5).

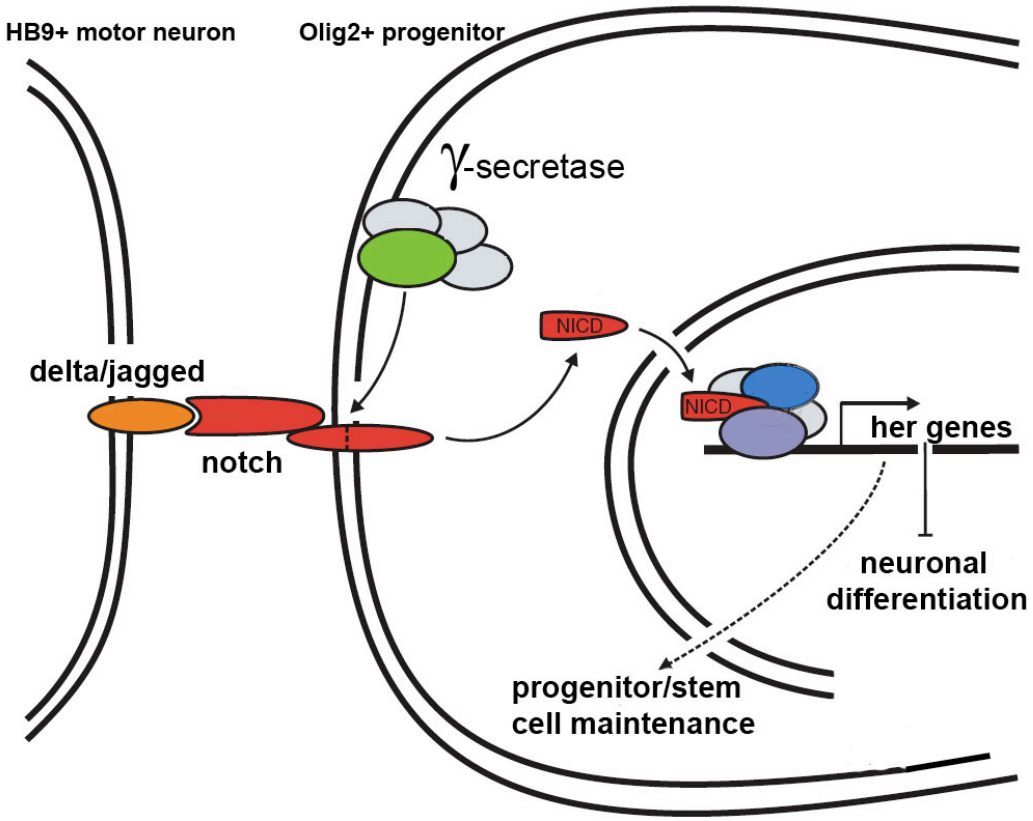

Fig 1.4 Schematic diagram of the Notch pathway. Notch signalling is a cell-cell communication process where HB9+ differentiating motor neurons in the zebrafish may express the delta/jagged family of ligands and bind the Notch receptor on Olig2+ progenitors, thus activating Notch signalling. Transcription of downstream her genes block neuronal differentiation and maintain the progenitor pool. Image modified from (Yoon and Gaiano, 2005).

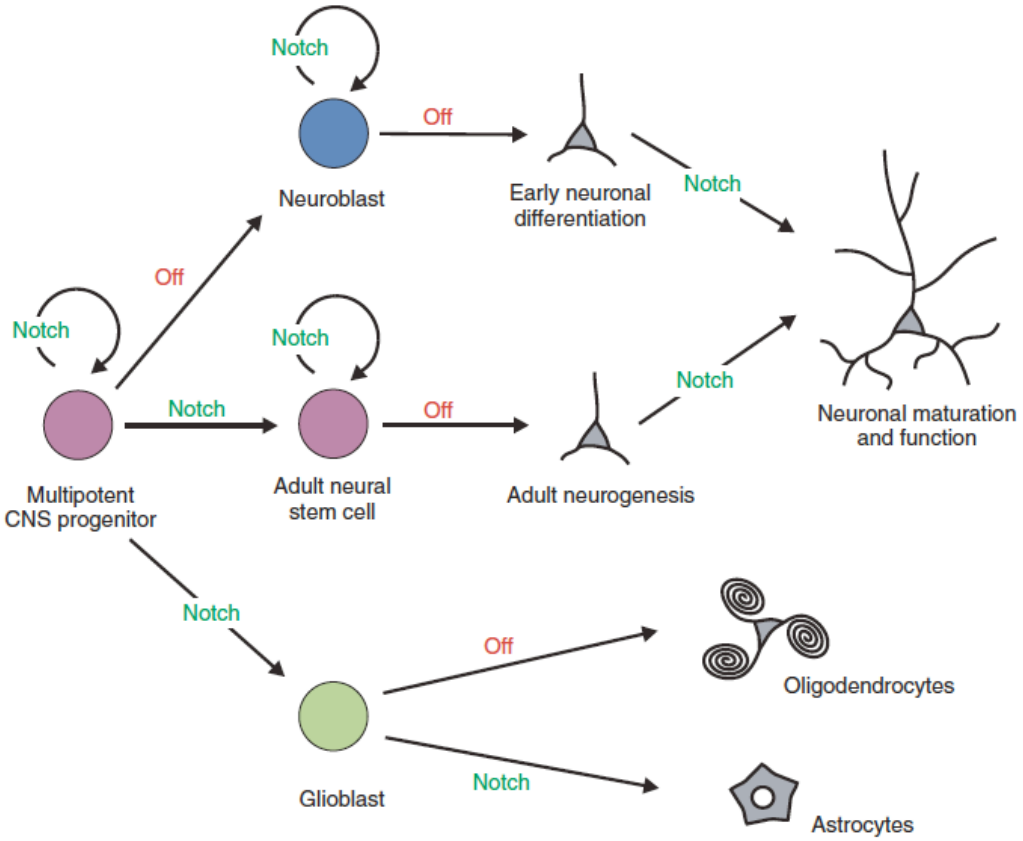

Fig 1.5 Role of Notch signalling in the developing and adult vertebrate central nervous system. Cell fate decisions that require Notch signalling are denoted as Notch (green) and those that do not require it are labeled as Off (red). Image taken from (Yoon and Gaiano, 2005).

1.5.1 Notch regulates neural cell fate in the developing and adult nervous system

The Notch pathway has been extensively studied in the fruit fly Drosophila Melanogaster nervous system where it controls cell fate choice in different contexts. Initially, the Notch pathway acts by selecting single cells to form neuroblasts from an equipotent cluster of progenitors resembling a "salt and pepper" pattern. The neuroblasts express basic helix-loop-helix (bHLH) transcription factors called proneural genes which are necessary and sufficient for their formation (Guillemot, 2007). In the vertebrate neural tube, neurons appear as

scattered cells intermingled with undifferentiated proliferative neuroepithelial progenitors and are reminiscent of the proneural clusters in the *Drosophila* neuroectoderm. The Notch pathway is involved in this selection process for neural progenitors that ultimately make neurons or glia as development proceeds (Lewis, 1996).

The premise that activating Notch would inhibit neuronal differentiation came from classic fly genetic studies which found that disruption of the Notch pathway led to precocious neuronal differentiation where all cells adopt a neural fate (Artavanis-Tsakonas et al., 1995). Conversely, when Notch signalling is constitutively active, all cells acquire an epidermal fate (Rebay et al., 1993). Together, these experiments and the identification of lateral inhibition in grasshopper embryos (Doe and Goodman, 1985) followed by observations in vertebrate cells of *Xenopus* and chick embryos showed that Notch activation influenced cell fate by blocking neuronal differentiation (Chitnis et al., 1995; Henrique et al., 1995). The well-known model of lateral inhibition consists of cells within a cluster communicating via an inhibitory feed-back loop of Notch signalling. Following several iterations of the loop only one cell in the cluster downregulates Notch signalling, expresses proneural genes and is thus chosen to become a neural precursor. Thus, Notch acts by inhibiting a specific differentiation program to choose between two fates (Cau and Blader, 2009).

During primary neurogenesis in the developing zebrafish spinal cord, multiple delta genes coordinate and mediate lateral inhibition in proneural clusters of neuroepithelial cells (Haddon et al., 1998). Similarly, in the mouse brain, Notch regulates the balance between progenitors and neuronal differentiation, supporting the lateral inhibition model (Kawaguchi et al., 2008). Mutations in the mammalian Notch1 or RBP-Jk genes (Recombination signal sequence Binding Protein for Jk genes) homologue of Su(H) leads to precocious neuronal differentiation at the expense of later born neurons (de la Pompa et al., 1997). It is important to note that

in vertebrates, Notch does not control the decision between epidermal versus neural fate as in the insect CNS but instead controls the timing of cell birth and subsequent differentiation. Consequently, Notch maintains proper timing of cell birth by preventing cell differentiation while maintaining a pool of progenitors.

Notch also serves as a negative regulator of both proliferation and neuronal differentiation, playing a role in the maintenance of the progenitor pool in the adult zebrafish forebrain (Chapouton et al., 2010; Chapouton et al., 2011). In the adult mouse telencephalon, ependymal cells proliferate after stroke and generate neuroblasts. However, disruption of Notch signaling leads to an exhaustion of the progenitor pool but the ependymal cells can be rescued by activating Notch (Carlén et al., 2009). Notch is also required for the maintenance of adult hippocampal neurons (Ables et al., 2010).

Newly generated post mitotic neurons in the zebrafish developing spinal cord express Delta ligands, which initiate Notch activity in the neighbouring cells. In this manner, lateral inhibition mediated by Delta-Notch signalling maintains the proliferative progenitor pool and is necessary for the generation of late-born neurons and glia (Appel and Eisen, 1998; Appel et al., 2001). Functional studies reveal that overexpression of deltaD mRNA in the zebrafish spinal cord leads to a reduction of primary motor neurons (Dornseifer et al., 1997; Appel and Eisen, 1998; Haddon et al., 1998). Conversely, deadly seven (des)/Notch1a mutant zebrafish identified in a genetic screen (van Eeden et al., 1996) show increased numbers of primary motor neurons (Gray et al., 2001). These observations further support the view that Delta-Notch signalling is necessary for coordinating cell cycle exit, differentiation of primary motor neurons and maintaining the progenitor pool.

Given that the Notch pathway influences cell fate in the developing spinal cord prompted us to investigate if the pathway is recapitulated in the regenerating adult zebrafish spinal cord. We hypothesize that if the Notch pathway was active then

newly generated HB9+ motor neurons (Reimer et al., 2008) would express the delta/jagged Notch ligands and communicate via Notch receptors on Olig2+ progenitor cells, upregulating the expression of her4, a downstream target gene (Figure 1.4). As mentioned above, the Notch pathway controls the timing of cell birth by serving as a negative regulator of neurogenesis while maintaining the progenitor pool in the developing and adult zebrafish CNS. If Notch signalling plays a similar role during motor neuron regeneration in the adult lesioned spinal cord then functional manipulations of Notch activity would provide an insight into its mechanism of action. We predict that constitutive expression of Notch will inhibit motor neuron generation and drive spinal progenitors back into quiescience. Conversely, inhibition of Notch signalling should force all spinal cord progenitors to differentiate into motor neurons.

1.6 Summary

In the mammalian spinal cord, regenerative mechanisms after spinal cord injury are poor. This is due to the inhibitory environment around the lesion site and possibly the Notch pathway. Zebrafish are an excellent model to study spinal cord regeneration because they can regrow their axons and replace damaged neurons. It is therefore, worthwhile to study the molecular mechanisms required for successful spinal cord regeneration, so that we can uncover ways to improve regeneration in mammals. In this dissertation, I have investigated the role of Notch signalling in the regenerating spinal cord of the adult zebrafish in Chapter 3. I find that if the levels of Notch are increased in the lesioned spinal cord motor neuron generation and proliferation is attenuated. Remarkably, this result is similar to the effect of Notch in the injured mammalian spinal cord (Yamamoto et al., 2001). Conversely, if we block Notch activity using DAPT, a γ-secretase inhibitor, we increase progenitor proliferation and the number of motor neurons generated. Thus, Notch acts as a negative regulator of progenitor proliferation and motor neuron regeneration.

While Notch activity in the lesioned spinal cord attenuates proliferation and the generation of motor neurons, an incidental find using the same manipulation prompted us to examine the role of Notch in the uninjured adult zebrafish retina in Chapter 4. I find that expression of Notch activity in the undamaged retina induces a massive increase in eye size and proliferation of presumptive Müller glia in the inner nuclear layer of the retina. It is tempting to speculate that Notch induces proliferation of Müller glia that then generate new born retinal neurons in the central retina. The over-activation of Notch did not have an effect on proliferation in the constitutively active ventricular zone of the forebrain. In addition, inhibition of Notch using DAPT did not have an effect on proliferation in the retina.

Finally, in Chapter 5 of this dissertation, I have used in situ hybridisation to assess gene expression patterns for RA genes that have previously been found to be upregulated after a lesion by PCR (Reimer et al., 2009). I find that the downstream genes of the RA pathway cyp26a and crabp2a are expressed in ventricular progenitor cells and in the region close to vicinity of the progenitor zone respectively. It has shown that brain-derived dopamine influences spinal progenitors to generate motor neurons (Norris et al., in revision). To determine which receptor dopamine is likely to signal through, I used in situ hybridization to determine the expression of all D2-like dopamine receptors in the adult lesioned spinal cord. I find that only the D4a receptor is upregulated all around the progenitor zone in the rostral half of the lesioned spinal cord but caudal to it.

Thus, RA may be involved in motor neuron generation after a spinal lesion and descending axons from the brain secrete dopamine that likely acts via the D4a receptor to influence the generation of motor neurons.

2. Materials and Methods

2.1 Materials

2.1.1 Enzymes

Restriction endonucleases:

- Various (5-20 U/µl)

New England Biolabs UK Ltd (Hitchin Hertfordshire, UK)

DNA polymerases:

- Taq DNA Polymerase with standard buffer

New England Biolabs UK Ltd (Hitchin Hertfordshire, UK)

- Go Taq

Promega (Southampton, UK)

- Moltaq Polymerase

Molzym GmbH & Co. KG (Bremen, Germany)

RNA-dependent DNA Polymerase:

- SuperScript®III (RT)

Invitrogen Ltd (Paisley, UK)

DNA-dependent RNA polymerases:

- MEGAscript™Kit SP6, T7, T3

Ambion, Applied Biosystems (Warrington, UK

Miscellanous:

- RNaseOUT™ Recombinant

Invitrogen Ltd (Paisley, UK)

Ribonuclease Inhibitor

- Random primer (500ug/ml)

Promega (Southampton,UK)

- RNaseZAP®R2020

Sigma-Aldrich (Dorset, UK)

- Proteinase K, recombinant PCR grade

Roche Diagnostics Ltd. (Burgess Hill, UK

2.1.2 Bacterial media and bacterial strains

All bacterial media were autoclaved before use. If necessary, Ampicillin antibiotic was added.

- Bacterial growth media encapsulated media LB medium

QBIOgene, Fisher Scientific (Loughborough, UK)

- LB Agar Miller Fisher BioReagents

Fisher Scientific (Loughborough, UK)

Bacterial strains:

- XL1-Blue competent cells

Stratagene (UK)

- NEB Turbo Competent *E. coli* (High Efficiency)

New England Biolabs UK Ltd. (Hitchin, Hertfordshire, UK)

- E. Coli DH5α

Invitrogen Ltd (Paisley, UK)

Antibiotics:

Ampicillin (50mg/ml in H2O stock, 50µg/ml working solution)

2.1.3 Vectors

- pGEM®-T easy

Promega (Southampton, UK)

2.1.4 Kits

- RNeasy® Mini Kit

Qiagen (Crawley, UK)

- Rapid DNA Ligation Kit

Roche Diagnostics Ltd (Burgess Hill, UK)

- pGEM®-T easy vector system I

Promega (Southampton, UK)

- QIAquick™ PCR Purification Kit

Qiagen (Crawley, UK)

- MinElute™ Gel Extraction Kit

Qiagen (Crawley, UK)

- GFX™ Micro Plasmid Prep Kit

GE Healthcare (Little Chalfont, UK)

- peqGOLD Plasmid Miniprep Kit I

PEQlab Ltd (Salisbury Green, UK)

- HiSpeed® Plasmid Midi Kit

Qiagen (Crawley, UK)

2.1.5 DNA Standards

- TrackIt™ 1 Kb Plus DNA Ladder

Invitrogen Ltd (Paisley, UK)

- 100bp DNA Ladder

New England Biolabs UK Ltd. (Hitchin, Hertfordshire, UK)

2.1.6 Oligonucleotides

- Primers (purification: RP-Column)

VH Bio Ltd. (Gateshead, UK) Integrated DNA technologies (Leuven, Belgium)

Table 1: Primer sequences used for PCR

Gene	Primer pair
GAPDH	F: 5' -ACTCCACTCATGGCCGTT- 3' R: 5' -TCTTCTGTGTGGCGGTGTAG- 3'
Her4.1	F: 5' -GGCTCAAGAGTTCGTCAAGC- 3' R: 5' -AGACGTGTGTGCTTGTCTGC- 3'
D4a	F: 5' -GCCTCTTTCCCATCTCACAG- 3' R: 5' -GCGCTGGGAAGTTGTAGTTC- 3'
D4b	F: 5' -GCCCGCGCTCTTCACTTAC- 3' R: 5' -CTGCCACAGCCAAACTCACG- 3'
D4rs	F: 5' -GTTTAAACCAGGTGCCGAACAGG- 3' R: 5' -ATACACCACCTTCTCCCTTGCC- 3'

Table 2: Primer sequences for cRNA probe synthesis

Gene	Primer pair
Jagged1a	F: 5' -TCGAGATCGAGGAGTGTGTCG- 3' R:5' -GGAGCTGTTAGCACTGTGTTTGC-3'
Her4.5	F:5'-ACACAAGACACACAGCAATGGC- 3' R:5'-GCCGTGTGGTCATCGTATAGACG- 3'
Her12	F: 5' -CTGTTCGAGCACAGACATGGC- 3' R: 5' -AATCCACCTCCTTCCCAGACG- 3'
D2b	F: 5' -AGCTCATGGATTTCCTCACG- 3' R: 5' -TTGGTTTCTTTTGGGGACTG- 3'
D4b	F: 5' -TCATCTGTGGAAACGTGCTC- 3' R: 5' -GCCATGTCACTGTGCTCATC- 3'
Raldh2	F: 5' - GAGCCCATTGGAGTGTGTGG- 3' R: 5'-CAGCCTATGAGGATGCAGCAG- 3'
Rarab	F: 5' - CCCATCAGGAAACCTTCCCC- 3' R: 5' - GAGAGAACGCGTCCTTCAGAC- 3'
RXRγa	F: 5' - CCCATCCATCATCAACGGTCT- 3' R: 5' - GCTTCCAGCATCTCCATGAGG- 3'
RXRγb	F: 5' - CTGTGCCATCTGTGGAGACC- 3' R: 5' - TTGCCACAATGGATACCTGGA- 3'

Cyp26a	F: 5' - GATCCGAACTGCAGAAGTCCT- 3' R: 5' - CACACACATCCTGGATCCTCC- 3'
Crabp2a	F: 5' - TCAAGACGTCCACCTCAGTG- 3' R: 5' - CAGGCACAAGACGTCAACAG- 3'

2.1.7 Antibodies

All primary antibodies listed in Table 3 were used in this study.

Table 3: Primary antibodies

Antigen name	Species	Source	Catalogue number/ Clone	Dilution
Digoxigenin-Alkaline Phosphatase,Fab fragments	Sheep	Roche	11093274910	1:10,000
GFP	Chicken	Invitrogen (Paisley, UK)	A11122	1:200
HB9 / MNR2	Mouse	Developmental Studies Hybridoma Bank	81.5C10	1:400
PCNA	Mouse	Dako Cytomation (Glostrup, Denmark)	PC10	1:1000
c-Myc (9E10)	Mouse	Santa Cruz Biotechnology	sc-40	1:600
c-Myc-FITC	Mouse	Santa Cruz Biotechnology	E-3007	1:1000

Secondary antibodies were purchased from Jackson Laboratories (Stratech, Newmarket, UK) and were used in a concentration of 1:200

Table 4: Secondary antibodies

Cy™2-conjugated Donkey Anti-Chicken IgG(H+L)	703-285-155
Dylight 488 Donkey Anti-Chicken IgG(H+L)	703-485-155
Cy™2-conjugated Goat Anti-Mouse IgG(H+L)	115-225-003
Cy™3-conjugated Donkey Anti-Mouse IgG(H+L)	715-165-151

Table 5: Plasmids for probe making

Gene	Promoter	Restriction site for linearisation	Origin
notch1a	T7	XbaI	(Bierkamp and Campos-Ortega, 1993)
notch1b	T3	BamHI	Julian Lewis (Leslie et al., 2007)
notch2	T7	BamHI	Ajay Chitnis (Lorent et al., 2004)
notch3	T7	HindIII	Ajay Chitnis

j1a	SP6	NcoI	Tatyana Dias
j1b	T3	BstXI	Ajay Chitnis (Yeo and Chitnis, 2007)
j2	T3	XbaI	Ajay Chitnis (Yeo and Chitnis, 2007)
deltaA	T7	EcoRI	Julian Lewis (Haddon et al., 1998)
deltaB	T7	EcoRI	Julian Lewis (Haddon et al., 1998)
deltaC	T7	XbaI	Julian Lewis (Smithers et al., 2000)
deltaD	T7	EcoRI	Julian Lewis (Haddon et al., 1998)
dll-4	T7	XhoI	Julian Lewis (Leslie et al., 2007)
her1	T7	BamHI	Julian Lewis (Giudicelli et al., 2007)
her3	SP6	NotI	Laure Bally-Cuif
her4.1	T7	SalI	Ajay Chitnis (Yeo and Chitnis, 2007)
her4.5	SP6	SphI	Tatyana Dias

her5	T3	XhoI	Laure Bally-Cuif (Chapouton et al., 2011)
her7	SP6	SalI	Laure Bally-Cuif
her8a	SP6	EcoRV	Laure Bally-Cuif (NM_199624)
her9	SP6	SalI	Laure Bally-Cuif (Leve et al., 2001)
her11	SP6	SacII	Laure Bally-Cuif
her12	SP6	SphI	Tatyana Dias
her13.1	SP6	EcoRV	Laure Bally-Cuif (XM_696748)
her13.2	T7	HindIII	Laure Bally-Cuif hes6 (NM_194400)
rarab	SP6	SacII	Tatyana Dias
rxrγa	SP6	SacII	Tatyana Dias
rxrγb	SP6	SacII	Tatyana Dias
cyp26a	SP6	SacII	Tatyana Dias
crabp2a	T7	SpeI	Tatyana Dias
D2a	T7	NotI	Boehmler et al., 2004
D2b	SP6	SphI	Tatyana Dias
D2l	T7	BamHI	Boehmler et al., 2004
D3	T7	Not1	Boehmler et al., 2004

D4a	T3	NotI	Boehmler et al., 2007
D4b	SP6	SphI	Tatyana Dias

2.1.8 Buffers and Solutions

10X phosphate buffered saline (PBS)	4g KCl 4g KH2PO4 28.39g Na2HPO4 160g NaCl Make up to 2 liters with dH_2O Adjust to pH 7.4
Paraformaldehyde solution	Paraformaldehyde 1M NaOH 10X PBS
PBSTx	0.1% (v/v) Triton X 100 in 1X PBS
Sodium Citrate Buffer, 10mM	1.47g Sodium citrate 500ml 1X PBS Adjust pH to 6.0

Blocking buffer (whole mount immunohistochemistry)	1X PBS 1% (v/v) DMSO 1% (v/v) normal goat serum (NGS) 1% (w/v) BSA 0.7% (v/v) Triton-X 100
Blocking buffer (vibratome section immunohistochemistry)	2% (v/v) normal goat serum in PBSTx
PBST	0.1% (v/v) Tween 20 in 1X PBS
Hybridization Buffer	5ml formamide 2.5ml 20X SSC 10µl Tween 100µl yeast tRNA (100mg/ml) 2.38ml RNase free dH_2O 10µl heparin (50mg/ml)
Saline sodium citrate (SSC) buffer20X	3M NaCl 0.3M Na-citrate Adjust to pH 7.0

Blocking Solution (vibratome section situ hybridization)	1% blocking reagent (Boehringer/Roche) in 0.1% PBST Pre warm – Do not boil
NBT	0.7ml N,B-dimethylformamide 0.3ml RNase free water store at -20 °C
BCIP	50mg BCIP 1ml N,N-dimethylformamide anhydrous, store at -20 °C
Labeling solution	22.5µl of 50mg/ml NBT 17.5µl of 50mg/ml BCIP 5ml Alkaline Tris buffer Prepare fresh, protect from light
Tris-acetate-EDTA (TAE) buffer10X	2M Tris-acetate 100mM EDTA Adjust to pH 8.5
dNTP stock solution (100mM)	dATP, dCTP, dGTP, dTTP, 25mM each

2.1.9 Chemical reagents and products

Name	Company
24-Well Tissue culture treated multiwell plates for adherent cell lines	Greiner Bio-One (Stonehouse UK)
Acetic anhydride, ≥ 98%	Sigma-Aldrich (Dorset, UK)
Agarose	Fisher Scientific (Loughborough, UK)
Cover glass, 24x50mm	VWR (Leicester, UK)
Disodium phosphate (Na_2HPO4)	Sigma-Aldrich (Dorset, UK)
Donkey Gamma Globulin (017-000-002)	Jackson Immuno Research Laboratories, Stratech (Newmarket, UK)
eSHA-2000	eSHA lab (Maastricht, NL)
Ethanol	Sigma-Aldrich (Dorset, UK)
Ethidium bromide	Fisher Scientific (Loughborough, UK)
Formamide	Sigma-Aldrich (Dorset, UK)
Glycerol	Sigma-Aldrich (Dorset, UK)
Glycine	Sigma-Aldrich (Dorset, UK)
Heparin sodium salt	Sigma-Aldrich (Dorset, UK)

Histoacryl	Braun (Tuttlingen, D)
Methanol	Sigma-Aldrich (Dorset, UK)
Microscope slide, superfrost plus	VWR (Leicester, UK)
NaOH	Sigma-Aldrich (Dorset, UK)
NBT/BCIP tablets	Sigma-Aldrich (Dorset, UK)
Needles, 0.3x13mm	BD MicrolanceTM3 (Drugheda, Ireland)
Normal Goat Serum	Millipore (Oxford, UK)
Normal Sheep Antibody	Covance (Alnwick, UK)
Potassium chloride (KCl)	Sigma-Aldrich (Dorset, UK)
Potassium disulphate (KH_2PO4)	Sigma-Aldrich (Dorset, UK)
Purified Agar	OXOID (Hampshire, UK)
Ribonucleic acid from torula yeast, Type VI	Sigma-Aldrich (Dorset, UK)
Sodium Chloride (NaCl)	Fisher Scientific (Loughborough, UK)
Sodium Citrate	Sigma-Aldrich (Dorset, UK)
Syringes, 1ml, 10ml	Plastikpak (Verbania, Italy)
Triton-X 100	Sigma-Aldrich (Dorset, UK)
Tween 20	Sigma-Aldrich (Dorset, UK)
Yeast t-RNA	Roche (Burgess Hill, UK)

2.1.10 Equipment

Name	Company
Balance	Acculab Sartorius group (Massachusetts, US)
Bench-top centrifuges 5417 R and 5804 R	Eppendorf (Cambridge, UK)
Centrifuge RC 5C Plus Sorvall	Kendro (Hanau, D)
Centrifuge Sigma 3K30C	Sigma Laborzentrifugen GmbH (Osterode am Harz, D)
Cryostat CM3050	Leica (Bensheim, D)
E.A.S.Y. UV-light documentation	Herolab (Wiesloh, D)
Fishsystem	Aqua Schwarz (Goettingen, D)
Fluorescence Microscope	Zeiss (Goettingen, D)
Hotplate stirrer Fisherbrand® metal top	Fisher Scientific (Loughborough, UK)
Hybridizer UVP HB-1000	Jencons PLS (East Grinstead, UK)
Incubated shaker MaxQ Mini 4450	Fisher Scientific (Loughborough, UK)
Laser scanning microscope LSM510	Zeiss (Goettingen, D)

Laser scanning microscope LSM710	Zeiss (Goettingen, D)
Microcentrifuge 5415 D	Eppendorf (Cambridge, UK)
MJ mini gradient thermal cycler	Biorad (Hempstead, UK)
NanoDrop ND1000,	Thermo Scientific (Surrey UK)
pH Meter HP220	Mettler-Toledo (Leicester, UK)
Pipettes	Eppendorf (Cambridge, UK)
Spectrophotometer Ultrospec 3000/DPV	APB (Freiburgh, D)
Stereomicrocope, KL 1500 LCD	Zeiss (Goettingen, D)
Sub-Cell GT / Power Pac Basic System	Biorad (Hempstead, UK)
Technico Mini centrifuge	Fisher Scientific (Loughborough, UK)
Vibratome Microm HM-650V	Optech Scientific Instruments (Oxford, UK)
Waterbath	Fisherbrand (Loughborough, UK)
Wide Mini-Sub Cell GT / Power Pac Basic System	Biorad (Hempstead, UK)

2.2 Methods

2.2.1 Molecular Biology techniques

2.2.1.1 Quantification, analysis and sequencing of nucleic acids

Quantification of nucleic acids

The purity and concentration of cDNA, dsDNA and RNA were measured using the NanoDrop (NanoDrop ND1000, Thermo Scientific, USA).

Agarose gel electrophoresis for DNA samples

Horizontal agarose gel electrophoresis was performed to analyze the outcome of restriction digests, PCR reactions and the quality of DNA samples. Depending on the size of fragments to be separated, gels were prepared by heating 0.8%-2.5% (w/v) of agarose (Fisher Scientific, UK) in 150ml 1X Tris-acetate buffer (TAE). After the agarose had dissolved in the buffer and cooled for 1 min, ethidium bromide (Fisher Scientific, UK) was then added (10µl/150ml), mixed well and poured into a gel caster. To load the samples on the gel, DNA loading buffer (Eppendorf, UK) was added to the DNA samples to a 1X fold final concentration. Electrophoresis was performed at 10V/cm in a BIO-Rad gel chamber in 1X TAE buffer. Gel results were documented using the E.A.S.Y. UV-light documentation system. For subsequent TA cloning, the DNA bands were visualized using a UV-screen (λ=360nm) and the desired fragments were cut out with a scalpel. The MiniElute™ Gel Extraction Kit (Qiagen, UK) was used to extract DNA fragments from the agarose gels, according to the manufacturer´s protocol.

DNA sequencing

Sequence acquisition and analysis of cDNA, plasmid DNA or PCR products was performed by DNA Sequencing & Services™ (University of Dundee, UK).

2.2.1.2 First strand cDNA synthesis

cDNA synthesis was carried out using random primers, the SuperScript III™ Reverse Transcriptase (RT) kit and the RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen Ltd., Paisley, UK) according to the manufacturer's protocol.

The synthesis steps were carried out in a MJ mini-gradient thermal cycler (Biorad, UK).

In 0.2ml PCR reaction tubes:

Total RNA	11µl
Random primers	1µl
dNTP mix (10mM)	1µl
Mix well and spin down	
In the cycler,	
5 min	65°C
1 min	place on ice, spin down
5X first strand buffer	4µl
DTT (0.1M)	1µl
RNaseOUT inhibitor	1µl
SuperScript III RT	1µl
Pipette to mix well, spin down	
In the cycler,	
5 min	65°C
60 min	50°C
15 min	70°C, store at -20°C

2.2.1.3 In-vitro RNA transcription

An in vitro transcription was performed using the MAXI-SCRIPT Kit (Ambion,

Cambridge, UK) to generate DIG labeled probes for in situ hybridisation. 10 µg of plasmid DNA containing the desired insert, flanked by a T7, SP6 or T3 promoter were digested with restriction endonucleases overnight. Thus, only the promoter sequence and the desired DNA insert were transcribed. The linearized plasmid DNA was precipitated as described above. For the generation of DIG labelled RNAs, DIG-11-dUTP (Roche, UK) was used. In-vitro transcription was performed using RNase free conditions. To avoid contamination with RNAase, RNAse-free filter tips and eppendorf tubes and RNase-free reagents were used. RNaseZAP was used to clean work surfaces. The reagents were thawed on ice for 30 min before use; the transcription buffer and ribonucleotides were vortexed to mix and briefly centrifuged. All reagents were assembled at RT.

Reagents for probe making:

10 mM ATP

10 mM CTP

10 mM GTP

6.5 mM UTP

3.5 mM DIG-11-dUTP

2 µL 10X Reaction Buffer

0.1–1 µg linear template DNA

2 µL Enzyme Mix

Up to 20 µL with Nuclease-free Water

37°C, 3 hr

2.2.1.4 Standard Polymerase Chain Reaction (PCR)

The standard PCR (Saiki et al., 1985), an amplification of DNA by in vitro

enzymatic replication, was performed in an MJ mini-gradient thermal cycler (Biorad, UK).

Reagents:

Template (cDNA)	10pg – 1ng
dNTPs	200µM (each dNTP)
Primer (forward)	0.1 – 1µM
Primer (reverse)	0.1 – 1 µM
Reaction buffer	(10X) 1X
DNA Polymerase	2.5U

add dH_2O to final volume 50µl

Program:

cycles	time	temperature
1	5 min	94 °C
	30 s	94 °C
25-40	45 s	Tm – 1 °C
	1 min per kb	72 °C
1	4 min	72 °C
	forever	4 °C

The PCR reaction was carried out in a 0.2ml PCR reaction tubes. Moltaq polymerase was often used to amplify DNA fragments upto 1.5kb long. GoTaq® green master mix (containing the Taq polymerase, dNTPs and reaction buffer) was

used to amplify transgenes from gDNA (obtained from fin clips – see below) for genotyping of adult zebrafish. After completion of the PCR reaction, 5µl - 10µl of the product was analysed by agarose gel electrophoresis, unless otherwise stated.

2.2.2 Molecular cloning

2.2.2.1 Taq DNA polymerase-amplified fragments

Using brain or whole embryo cDNA as a template, genes of interest were amplified using specific primers and Taq DNA polymerase in a standard PCR reaction as described above. The amplified DNA fragment was purified from PCR products using the MinElute™ PCR Purification Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol. The purified DNA fragment was eluted from the column with 50ul DNase free water and the concentration was measured using the Nano-drop. Subsequently, the purified fragment was subjected to TA cloning.

2.2.2.2 TA Cloning

TA cloning is a form of subcloning which does not require the use of restriction enzymes and exploits the terminal transferase activity of Taq polymerase. The enzyme preferentially adds a single, 3'-Adenosine overhang to each end of the amplified DNA fragment. Thus, it is possible to directly clone this PCR amplified insert into a pre-linearized cloning vector (pGEM®-T easy vector) that contains single 3'-Thymidine overhangs. The complementary base pairs (A & T) on the different DNA fragments hybridized under the action of a T4 DNA ligase. All procedures were carried out using the pGEM®-T easy vector system 1 (Promega, UK), following the manufacturer's instruction.

2.2.2.3 DNA transformation into bacteria

Plasmid DNA (10ng) or ligation mixture (5µl) was added to competent bacterial cells and gently inverted a couple of times to mix. This mixture was incubated for 30mins on ice. The bacterial cells were then heatshocked for 30 seconds at 42°C to enable them to take up the plasmid DNA or ligated vectors. Following heatshock,

the bacterial-plasmid mixture was incubated on ice for 5 min, 500µl of SOC-medium was added and subsequently incubated at 37°C for 1 hour at 200 rpm in an orbital shaker. The bacteria were then plated on LB agar plates containing the appropriate antibiotic, IPTG and X-gal for blue-white screening. The plates were sealed with parafilm and incubated upside down at 37°C overnight. Cells that are transformed with a plasmid containing an insert form white colonies, while others transformed with a plasmid but without an insert form blue colonies. White bacterial colonies were picked after 12-14 hours at 37°C.

2.2.2.4 Small scale plasmid extraction (Mini-prep)

A mini-culture was set up in a 15ml falcon containing 5ml LB medium with the required antibiotic, inoculated with a single colony and incubated at 37°C overnight at 200 rpm in an orbital shaker. 1.5ml of the mini-culture was transferred into a 2ml Eppendorf tube and the cells were pelleted by centrifugation (13,000 rpm, 1min, RT). The plasmid was extracted using the GFX™ Micro Plasmid Prep Kit (GE Healthcare, Little Chalfont, UK) or the peqGOLD Plasmid Miniprep Kit (PEQlab Ltd, Salisbury Green, UK) according to the manufacturer's protocol. Finally, the DNA was eluted from the column with 50µl DNase free water and the concentration was measured using the Nano-drop.

2.2.2.5 Medium scale plasmid extraction (Midi-prep)

To scale up the quantity of plasmid DNA, 1ml of a mini-culture (culture containing bacteria from a single colony, as described above) was incubated in 50ml of LB medium containing the appropriate antibiotic at 37°C overnight at 200 rpm in an orbital shaker. The midi-culture was transferred into a 50 ml falcon tube and the plasmid was extracted using the HiSpeed® Plasmid Midi Kit (Qiagen, UK), according to the manufacturer´s protocol. Finally, the DNA was eluted from the column with 500µl DNase free water and the concentration was measured using the Nano-drop.

2.2.2.6 Restriction enzyme digestion of plasmid DNA

Plasmid DNA extracted from the mini/midi-preps was analyzed using restriction enzyme digestion. The plasmids were digested with the appropriate amount of restriction endonucleases (NEB) according to the manufacturer's instructions. These control digestions were performed at the optimum temperature of the enzyme used for 3 hours in a 20µl reaction volume. For preparative digestions such as synthesizing full length cRNA probes from plasmid DNA, the restriction enzyme chosen was based on the sequence of the plasmid, orientation of the gene insert and restriction sites. The reaction volume was scaled upto 100µl and incubated overnight at the appropriate temperature to generate a linearised plasmid for probe synthesis.

2.2.2.7 DNA precipitation

Linearized DNA for probe synthesis was precipitated using the following protocol:

Sodium acetate (3M, pH 4.9, 1:10 v/v) and 2.5x volumes cold (-20°C) ethanol was added to the DNA. After mixing gently, the reaction tubes were centrifuged for 30 min at 13000rpm at RT. The supernatant was carefully removed and the pellet was washed with 900µl 70% ethanol. After being centrifuged for another 5 min, the supernatant was removed and the pellet was left to dry for 30 min at RT and resuspended in 20µl RNase free dH_2O. The tubes were then kept at 4°C for 30 min to allow re-hydration of DNA. The linearized DNA was then stored at -20°C. The concentration of the linearized DNA was checked with a NanoDrop (ND1000, Thermo Scientific, USA).

2.3 Animal experiments

2.3.1 Fish husbandry and maintenance

Zebrafish (*Danio rerio*) were kept at a temperature of 26.5°C on a 14-hour light and 10-hour dark cycle. Larval and adult fish were fed with dry flakes, ZM pellet food (ZM Ltd., UK) and *Artemia salina* larvae (ZM Ltd., UK). Adult fish were

bred and larval fish were raised according to standard protocols (Westerfield, 1989). All experiments were licensed by the British Home Office.

We used wildtype (Curtis et al.), *Tg(olig2:EGFP)* (Shin et al., 2003), hereafter designated olig2:GFP, *Tg(mnx1:GFP)ml2 (Flanagan-Steet et al., 2005)*, hereafter designated HB9:GFP, *Tg(hsp70l:Gal4)* and *Tg(UAS:myc-notch1a-intra)* (Scheer et al., 2001) transgenic fish, as well as *notch1a* mutants (*notch1a*$^{tp37/tp37}$) (van Eeden et al., 1996).

2.3.2 Genotyping using fin clips

Adult notch1a mutant and Tg(hsp70l:Gal4) X Tg(UAS:myc-notch1a-intra) zebrafish from > four months to one year were used for fin clipping to determine the appropriate genotype. Fish were anesthetized in aminobenzoic acid ethylmethylester (MS222) in PBS (1:5000; Sigma, St. Louis, MO) and then placed on a damp paper towel on a metallic board with the tail fin neatly fanned out. Then 3-5mm from the tip of the tail was cut off using a cutthroat blade and placed into an appropriately labelled 0.2ml PCR tube on dry ice using an insect pin. The fish were then placed in tanks (with the same label as the PCR tubes) containing 800µS high salt water to prevent any bacterial or fungal infections and returned to the re-circulating system the next day following genotype analysis.

All subsequent steps were performed in a MJ mini-gradient thermal cycler (Biorad, UK). For extraction of gDNA the fin clips were incubated at 98°C for 10min in 50µl of lysis buffer making sure that the fins were at the bottom of the PCR tubes. Then 10µl of proteinase K at 10µg/ml was added and the tubes were incubated at 55°C overnight. The tubes were then maintained at 98°C for 10 mins to denature the proteinase K, stopping its activity. The gDNA was diluted at 1:20 in nuclease free water and used as template with the appropriate primers to amplify the transgene of interest. The PCR reaction was carried out using the standard method as described before.

2.3.3 Spinal cord lesions and injections

Adult zebrafish from > four months to one year were used for spinal cord lesions. Zebrafish were housed in single tanks with 1300µS high salt water for 48 hours before performing the spinal cord lesions. This was done to allow them to adapt to a high salt concentration. After the surgery, lesioned fish were kept in the high salt water to minimize any risk of infection. It is essential to avoid additional stress after surgery hence adaptation is required. As described previously (Becker et al., 1997) fish were anesthetized in aminobenzoic acid ethylmethylester (MS222) in PBS (1:5000). For the duration of the surgery, the fish were kept on ice for further analgesia and constriction of blood vessels in order to minimize any bleeding. The vertebral column was exposed by making a longitudinal incision at the side of the fish to create a surgical window. A complete transection of the spinal cord was done under visual control 4 mm caudal to the brainstem-spinal cord junction. The resulting wound was closed with Histoacryl (Braun, Melsungen, Germany). Lesioned fish were kept in single tanks with 1300µS high salt water with ESHA2000, in the dark for seven days to avoid any unnecessary stimuli and to facilitate the healing process.

For substance injections, fish were anethetized as described above. DAPT (*N*-[*N*-(3,5-difluorophenacetyl-l-alanyl)]-*S*-phenylglycine *t*-butyl ester) (Enzo Life Sciences, Exeter, UK) was injected intraperitoneally, delivering a dose of 50mg/kg body weight in a volume of 25µl at 3, 6 and 9 days post lesion (dpl). Analysis took place at 14dpl. To test the effect of DAPT in the unlesioned spinal cord, fish were injected with DAPT intraperitoneally for 5 consecutive days. Analysis took place 8 days post injection.

2.3.4 Heatshock administration

All heatshocks were administered in Tg(hsp70l:Gal4) X Tg(UAS:myc-notch1a-intra) zebrafish embryos or adult fish. Using the Gal4/UAS system, double

transgenic heatshocked fish are capable of overexpressing the Notch intracellular domain under the control of the heatshock promoter. The Notch intracellular domain is Myc-tagged (NICD-MYC) and can be visualized using anti-Myc immunohistochemistry.

2.3.4.1 Heatshocks in embryos

Double transgenic zebrafish embryos at 24 hours post fertilisation were placed in a glass beaker containing 120ml autoclaved fish water. The opening of the beaker was closed with parafilm. The beaker containing the embryos was placed in a waterbath preheated to a temperature of 39°C and the embryos were heatshocked for 40 minutes. After the heatshock, the embryos were transferred to a petri dish containing autoclaved fish water and placed in a water bath maintained at 28.5°C. The embryos were allowed to develop for one hour to allow overexpression of the Notch intracellular domain. Analysis was at ~ 26 hours post fertilisation.

2.3.4.2 Heatshocks in adult lesioned fish

Spinal cord lesions on adult double transgenic fish were performed as described above. The lesioned fish were transferred to a stand alone aquarium with 1300µS high salt water at 4dpl. Heat-shocks were delivered daily from 5dpl to 13dpl for 1 h by slowly (approx.2 h) heating the tank water from 26°C to 39°C. Water temperature was allowed to decrease gradually. Analysis was at 14dpl. Lesioned fish were heatshocked as aforementioned at 12dpl and 13dpl with analysis at 14dpl to visualize the NICD-MYC in the lesioned spinal cord.

2.3.4.3 Heatshocks in adult unlesioned fish

To test the effect of Notch overexpression in the unlesioned spinal cord, double transgenic adult fish were housed in single tanks with 1300µS high salt water for 48 hours before administering heatshocks. This was done to allow them to adapt to a high salt concentration. Unlesioned fish were then transferred to the stand-alone tank with 1300µS salt water. Heatshocks were administered as described above for

2, 5 or 9 consecutive days with analysis at 3, 6 or 10 days post heatshock respectively.

2.3.5 Extraction of total RNA from zebrafish tissue

Total RNA was isolated from whole embryos, brain and spinal cord. Fish were killed via a schedule 1 method (Home Office, UK) and the tissue of interest was micro-dissected quickly and processed for RNA isolation using the RNeasy® Mini Kit (Qiagen, Crawley, UK) according to the manufacturer´s protocol. The total RNA was eluted from the column in 30µl RNase free water. The amount and quality of total RNA extracted was measured using the Nano-drop. RNA samples were stored at -80°C. To assess the effect of Notch manipulations on the downstream target gene her4.1, spinal cord tissue from lesioned fish was collected from the area of 3 mm surrounding the lesion site (rostral and caudal). The same amount of spinal cord tissue was removed from unlesioned controls. Tissue from at least 5 animals per treatment was pooled together before RNA isolation. For the DAPT experiment, tissue was collected at 5dpl from fish that had received a single injection of DAPT at 4dpl. For the Notch overexpression experiment, tissue was collected at 10dpl from fish that had been heatshocked from 5dpl to 9dpl.

2.3.6 Tests of swimming capability

The endurance of lesioned fish that were either DAPT or vehicle treated was tested by recording the time they were able to hold their position in a water flow (15cm/s), as previously published (Reimer et al., 2009) with the exception that the test duration was limited to 1 hour. The spontaneous movements of fish during a 5 minute period in standing water was also recorded using an overhead camera and Ethovision (Tracksys, Nottingham, UK) software, as reported (Becker et al., 2004).

2.3.7 Tissue fixation in embryos

Washing steps were performed at room temperature (RT) for 5 minutes (min) each time, unless stated differently. For subsequent immunohistochemistry, 24hpf zebrafish embryos were dechorionated and de-yolked in autoclaved fish water with watchmaker's forceps and metal needles. The embryos were transferred to a petri dish containing 4%PFA with 1% (v/v) DMSO and fixed for 45 minutes at RT. The embryos were then washed 3X with 1X PBS. (Embryos can be stored for one week).

For in situ hybridisation, 24hpf embryos were dechorinated and with the yolks left intact they were fixed in 4% paraformaldehyde in 1X PBS at 4°C overnight. The embryos were washed 3x with PBS/0.1% Tween-20 (PBST) and 1x with 100% methanol. After transferring the embryos into fresh 100% methanol, fixed embryos were stored at –20°C overnight before use. (Embryos can be stored for several months).

2.3.8 Tissue fixation in adult zebrafish

Adult zebrafish were transcardially perfused as described below for all in situ hybridization and immunohistochemical stainings. Adult fish were anesthetized using MS222 diluted in 1X PBS (1:1000). On exposing the heart, the saccus vasculosus attached to it was cut disrupting blood circulation. The blood was flushed out using 1X PBS by inserting a glass needle attached to a syringe (by a plastic tubing) through the heart into the bulbus arteriosus. The syringe was then replaced with a syringe containing 4% paraformaldehyde and 3ml/3min was flushed through the fish to fix the tissue.

2.4 Histological techniques

2.4.1 Immunohistochemical staining in floating sections

Immunohistochemistry on 50µm vibrating blade microtome sections was performed as previously described (Reimer et al., 2009). Washing steps were performed at RT for 15 minutes (min) each time, unless stated differently. Brain, Spinal cord and retinae were microdissected and embedded in 4% purified agar in 1X PBS. Agar blocks were cut in the shape of pyramids and 50µm thick coronal sections were made using a vibrating blade microtome. The sections were collected in 1X PBS in 24-well plates. For nuclear labelings- HB9 and PCNA antigens were retrieved by incubating sections at 80°C in sodium citrate buffer (10mM sodium citrate in PBS, pH 6.0) for 30 min. After retrieving the antigen, sections were incubated in 50mM glycine in PBSTx for 10 min and washed 1x in PBSTx. Non-specific binding sites in the sections were blocked by incubating the sections in 1.5% normal donkey or goat serum (NDS/NGS) in PBSTx for 1 hour. The sections were incubated with one or two different primary antibodies diluted in NDS or NGS in PBSTx at 4°C overnight on a shaker.

Following incubation with the primary antibody, sections were washed 3x in PBSTx and incubated with secondary antibodies at RT for 1 hour. All secondary antibodies used were diluted at 1:200. Subsequently, sections were washed 3X in PBSTx and 1X in PBS. If required, sections were incubated in Hoechst dye in PBS (1:1000) to stain the nuclei and washed 2X in PBS. Finally, the sections were mounted in 70% glycerol/PBS. For GFP and Myc labelings, sections were washed 1x in PBSTx and then the standard protocol was followed from the glycine incubation right through to mounting the sections. In some instances, a directly coupled fluorescein-tagged anti-myc antibody was used. To control for specific reactivity of the secondary antibody, alternating sections were not incubated with the primary antibody.

2.4.2 Immunohistochemical stainings in whole mount embryos

To detect the NICD-Myc transgene expression pattern in 24 hpf embryos, whole-mount immunohistochemistry was performed. 24hpf zebrafish embryos were fixed as described before. Fixed embryos were incubated in blocking buffer at RT for 30min to prevent unspecific binding of the primary antibody. After blocking, embryos were incubated in primary antibody diluted in blocking buffer at 4°C overnight on a shaker. Any unbound primary antibody was washed 3x in 1X PBS. Embryos were then incubated with secondary antibody diluted in blocking buffer diluted 1:200 at 4°C overnight. Excess secondary antibody was washed 3x in 1X PBS. Finally, embryos were cleared in an ascending glycerol series – 30%, 50% and 70% glycerol in PBS. Embryos were mounted in 70% glycerol/PBS. In some instances, a directly coupled fluorescein-tagged anti-myc antibody was used.

2.4.3 *In situ* hybridization on 50µm thick vibrating blade microtome sections

The method for non-radioactive in situ hybridization on 50 µm sections has already been described (Lieberoth et al., 2003).

The work areas were kept RNAse-free and were cleaned with RNAaseZAP (Sigma-Aldrich). Solutions were made up with RNase-free water. If not stated otherwise, washing steps were for 5 min. All steps at 55°C were done in a waterbath. The spinal cord or brain was dissected out and embedded in 4% purified agar. 50µm thick sections were then made using a vibrating blade microtome and collected into 24-well-plates. The sections were washed 2x in PBST and then digested with proteinase K (Roche PCR Grade; 0.7µl/ml) for 9 min at RT. After washing 2x with glycine/PBST (2mg/ml), the tissue was postfixed in 4% PFA for 20 min. The sections were then washed 4x in PBST and washed 1x in 300µl whole mount hybridization buffer. After replacing with fresh whole mount hybridization buffer (500µl per well), the plate was wrapped with parafilm. The sections were

prehybridized for 3h at 55˚C. Digoxygenin labeled probes (1:1000 or 1:500 in whole mount hybridization buffer) were heated at 80°C for 10 min in the water bath. The probes were then briefly centrifuged and chilled on ice for 2 min. The prehybridization buffer was replaced by probe/hybridization buffer solution and the plates were wrapped with parafilm and incubated at 55°C overnight.

The following steps were done at 55°C. The solutions were preheated for 10 min. The sections were washed 2x in 50% formamide/2XSSC + 0.1% Tween for 30 min each, once in 1X SSC + 0.1% Tween for 15 min and 2x in 0.2X SSC + 0.1% Tween for 30 min each. The sections were then blocked for 1h in 10% (10g blocking reagent in 100ml PBST) Boehringer Roche blocking reagent at room temperature (500µl per well). Anti-DIG alkaline phosphatase coupled fab fragments (Boehringer/Roche) were diluted 1:2000 in blocking reagent (1:2000), added to the sections and left overnight at 4°C on a shaker. On the third day, the sections were washed 6x for 20 min each in PBST on a shaker and then once in PBS. Staining solution was prepared by dissolving one NBT/BCIP tablet (Sigma) in 10 ml dH_20. The sections were washed 1X in staining solution and after exchanging with fresh staining solution the sections were incubated for 30 min to overnight, depending on the kinetics of signal development. Once in situ signal had developed, the staining solution was removed and the sections were washed several times in PBS. If the sections were not being processed for subsequent immunohistochemical staining, 70% Glycerol/PBS was added and sections were mounted onto slides in 70% Glycerol/PBS. When the sections were processed for subsequent immunohistochemical staining, sections were washed several times in PBS before commencement of the immunohistochemical procedure. This was performed as described in (2.4.1), starting with the incubation step in 50 mM glycine. No antigen retrieval was done for non-nuclear immunolabellings, but for nuclear stainings the immunohistochemical procedure started at the sodium citrate incubation.

2.4.4 Whole mount *in situ* hybridization

If not stated otherwise, washing steps were for 5 min and performed at RT. Fixed embryos (2.3.7) were distributed into autoclaved eppendorf tubes (8-9 embryos per tube) and rehydrated a in methanol gradient - 75%, 50%, 25% methanol in PBST. The embryos were then washed 2x in PBST. The subsequent protocol was the same as the one used for the in situ hybridisation on vibratome sections up to the step where the staining solution is removed. After washing several times in PBS, embryos were either frozen for subsequent sectioning on the cryostat or cleared in an ascending gradient of glycerol with PBS (30%, 50% and 70% glycerol in PBS). Once they had sunk to the bottom, the next higher concentration of glycerol was used. Slides were prepared with 4 drops of vaseline as spacers and embryos were mounted in 70% glycerol/PBS.

2.5 Image acquisition and figure plates

For documentation of experiments, I used either a Zeiss LSM 510 or a Zeiss 750 LSM Confocal Microscope using 20x and 63x oil immersion objectives.

Images were processed with Zeiss LSM Image Browser and Image J. Fiqure plates presented in this thesis was done using Photoshop using raw data. For images of the eyes in Chapter 2, I used a stereomicroscope. For all pictures of in situ hybridisation signals, a 40X oil immersion objective was used.

2.6 Stereological counts

Small (nuclear diameter < 6 µm), intensely labeled HB9+ cells and ventricular PCNA+ cells were counted in a stereological fashion by an observer who was blinded to the treatments, as described (Reimer et al., 2008). Briefly, confocal image stacks of three randomly selected vibratome sections from the region up to 750µm rostral to the lesion site and three sections from the region up to 750µm caudal to the lesion site were analyzed. Cell numbers were then calculated for the

entire 1.5 mm surrounding the lesion site. For counts in the retina, PCNA immunoreactive cells in an area of 920µm from the circumferential germinal zone (CGZ) were counted.

2.7 Profile counts

For relative values, all Hoechst dye (Hoechst 33342, Biotium) labeled cells around the ventricle was counted in single optical sections to indicate the total number of ventricular cells. PCNA and/or myc labeling was determined in the same sections and values were expressed as percentage of all Hoechst-labeled cells. At least 18 optical sections were used from 6 physical sections within 1500µm of the lesion site per fish.

2.8 Statistical analysis

Cell numbers were analyzed using the Mann-Whitney U-test if not indicated differently and behavioral recovery was tested using the two-way ANOVA test, followed by Tukey post-test. Cell numbers for PCNA immunoreactivity in the retina were analysed using the Kruskal-Wallis followed by Dunn's post-test.

3. Notch signaling controls generation of motor neurons in the lesioned spinal cord of adult zebrafish

3.1 Introduction

Adult zebrafish have an amazing capacity to regenerate organs in response to injury such as their fins, lateral line, heart and brain. This striking and inherent regenerative potential is also clearly evident in the spinal cord. Adult zebrafish are capable of regenerating their spinal cord following a complete spinal cord transection at midthoracic level two, halfway between the dorsal fin and the operculum corresponding to the eighth vertebra. After surgery, lesioned fish appear to be paralysed caudal to the lesion site (Becker et al., 1997).

3.1.1 Degeneration in the spinal cord following injury

a) Axonal degeneration

Wallerian degeneration of severed axons in the caudal spinal cord is initially observed at 4dpl with the presence of collapsed axons and loose and contorted myelin sheaths (Becker and Becker, 2001; Kuscha et al., 2011). Terminals derived from descending tyrosine hydroxylase positive (TH1+) and serotonergic positive (5HT+) axons that innervate the caudal spinal cord are completely lost post injury (Kuscha et al., 2011). The lesion induces a macrophage/microglial response in the caudal spinal cord as detected by immunolabelling of spinal crossections with the 4C4 antibody, which reveal the presence of rounded cells that are reminiscent of macrophages or activated microglia. Phagocytic cells with inclusions of myelin debris from degenerating myelinated axons is also observed by electron microscopy at 2wpl (Becker and Becker, 2001).

b) Cellular degeneration

Axonal degeneration is accompanied by a loss of motor neurons. Newly differentiated motor neurons express the transcription factor HB9. On reaching

maturity along with successful synaptic connections they start to express the enzyme choline acetyl transferase (ChAT). In the unlesioned zebrafish spinal cord motor neurons are divided into two broad classes on the basis of cell diameter and expression of ChAT, a marker for terminal differentiation. Large diameter motor neurons are HB9+ and ChAT+, indicating that they are terminally differentiated. In contrast, small diameter motor neurons do not express ChAT. The numbers of large diameter motor neurons decrease from 133 ± 34.9 in unlesioned animals to 42 ± 15.1 in lesioned animals at 1wpl as seen by terminal deoxynucleotidyl transferase-mediated biotinylated UTP nick end labeling (TUNEL). Unlesioned animals have on average about 20 ± 7.7 small motor neurons (Reimer et al., 2008).

3.1.2 Regenerative response in the lesioned spinal cord

a) Axonal regeneration

Within 3 to 4wpl, the dorsal fin and at 6wpl the tail fin regain movement (Becker et al., 1997). The total distance swum by lesioned animals at 1wpl significantly decreases to 5% in contrast to a 57% increase in locomotor function at 6wpl as measured by an open-field behavioral test for locomotor function. It is important to note that most lesioned fish show some degree of behavioral recovery after the regeneration phase. However, some fish develop an “S” shaped body posture and are capable of moving only the rostral half of their bodies. Longer recovery periods do not lead to an improvement in swimming behavior. In addition, the incidence of recovery for smaller younger fish is better than that for larger older fish (Becker et al., 2004).

Anatomical regeneration of degenerated axons correlates with successful behavioral recovery (Becker et al., 1997; Becker et al., 2004). Sprouting of axons in the rostral spinal cord initiates at 2wpl with axons crossing over the lesion site into the caudal part. At this point, the beginning of an axonal bridge between the rostral and caudal halves of the spinal cord is visible. An electron microscopic

view of the lesion site at 6wpl reveals that the spinal architecture is not restored to its original form and contains unmyelinated and remyelinated regenerated axons. Adjacent to the lesion site, the spinal cord has normal cytoarchitecture except that white matter tracts are filled with myelin debris from degenerating fibers. Thus, eluding to the fact that the tissue existed before the injury to the spinal cord (Becker and Becker, 2001).

Anterograde tracing experiments demonstrate projections from 20 brain nuclei that project axons beyond the lesion site to the distal spinal cord at 6 weeks post injury (Becker et al., 1997). Mechanical blockade of the lesion site with Teflon tape stops descending axons from crossing over into the caudal spinal cord and thus impairs the locomotor response. Futhermore, morpholino knockdown of L1.1, an axonal growth promoting homolog of the mammalian recognition molecule L1, reduces axonal regrowth and also abates functional recovery (Becker et al., 2004). Rostral to the lesion site, 80% of regenerating axons of descending tracts extend into major white matter tracts as would be expected. Interestingly, anterograde tracing experiments show that 74% of the descending axonal projections in the caudal spinal cord regenerate in the spinal gray matter instead. They re-route preferentially due to the macrophage/microglial response in the caudal spinal cord (Becker, 2001 #642).

TH1+ and 5HT+ terminal varicosities in the rostral spinal cord increase in density and re-innervate the caudal half at 6wpl compared to unlesioned animals. The abundance of regrown TH1+ and 5HT+ terminals in the caudal spinal cord after a lesion correlates with successful functional recovery. Significantly, re-lesioning recovered fish eliminates regenerated TH1+ and 5HT+ terminals and subsequent swimming ability (Kuscha et al., 2011).

Only 50% of the original TH1+ and 5HT+ terminal densities are re-established in the caudal spinal cord. Furthermore, TH1+ and 5HT+ axons fail to re-innervate the far caudal levels of the spinal cord. Thus, it is clear that a significant amount of

axonal plasticity occurs in the caudal spinal cord after a lesion. Nevertheless, this does not affect swimming capability (Kuscha et al., 2011). The extent of axonal regrowth in the rostral and caudal spinal cord is probably sufficient for adapting the propagation of the rostrocaudal wave of excitation in the spinal network during swimming (Grillner et al., 2008).

b) Cellular regeneration

Regeneration is not a complete process and different populations of axotomized brain nuclei vary in their response to re-project their long range axons. A majority of axotomized neurons in brain nuclei upregulate the expression of GAP-43, a growth related gene. Only 50% of these neurons regrow descending axons. In relation to the regeneration of ascending tracts, 31% of spinal neurons upregulate GAP-43 of which 2 - 4% regrow axons to the brainstem. Interestingly, many uninjured neurons in the lesioned spinal cord upregulate GAP-43 indicating plasticity in the spinal circuitry (Becker et al., 2005).

During the reparative phase after the lesion, some spinal cell types regenerate within a similar time course as that seen for the regeneration of severed axons. However, various cell types have different regenerative responses following the spinal transection (Reimer et al., 2009; Kuscha et al., 2012). In the developing spinal cord, cellular identities for ventricular neuroepithelial progenitors is conferred by a gradient of the floor plate derived morphogen Shh and the combinatorial expression of bHLH homeodomain transcription factors. This two-dimensional system demarcates 7 dorsal and 5 ventral progenitor domains which gives rise to different cell types (Jessell, 2000; Vallstedt et al., 2001; Fuccillo et al., 2006). Similarly, in the lesioned spinal cord the progenitor cells maintain embryonic dorso-ventral positional information (Reimer et al., 2009). These ventricular cells contribute to the ependyma and contact the pial surface with glial endfeet. The lesion triggers massive proliferation of ventricular ependymo-radial

glial cells which steadily increases upto and peaks at 2wpl (Reimer et al., 2008). Small diameter and homeobox gene HB9 expressing motor neurons originate from proliferating Olig2:GFP+ progenitors in the pMN domain constituting the ventro-lateral zone of the lesioned spinal cord. The pMN progenitors receive a Sonic Hedgehog signal for motor neuron generation from ventral midline cells. Around 800 ± 106.8 HB9+ motor neurons are newly generated compared to 20 ± 7.7 in unlesioned animals at 2wpl (Reimer et al., 2008; Reimer et al., 2009). At 6wpl, the cell numbers of large diameter terminally differentiated ChAT+ motor neurons (91 ± 11.5) is not different from those in unlesioned animals. Thus, only 10% of the newly differentiated motor neurons fully mature and replace lost neurons. The remaining newly generated cells die as seen by their increased association with macrophage/microglial cells during the regenerative period upto 6wpl (Reimer et al., 2008).

Intraspinal 5HT+ neurons originate from a region that is ventral to the ventrolateral zone in the lesioned spinal cord. Numbers of 5HT+ neurons steadily increases up to 6wpl, when it is 5 fold higher than in unlesioned controls. At 13wpl, selective pruning reduces the total amount of 5HT+ neurons making the number more comparable to that seen in unlesioned animals (Kuscha et al., 2011). Undifferentiated V2 interneurons arise from the ventromedially located p2 progenitor domain, dorsal to the pMN domain in the embryonic spinal cord and express the transcription factor visual system homeobox 1 (vsx1) (Kimura et al., 2008). Following spinal lesion there is a 24-fold increase in the numbers of vsx1+ interneurons at 2wpl compared to unlesioned animals. At 6wpl, the number of V2 interneurons in the lesioned spinal cord is still high (Kuscha et al., 2012).

In contrast to vsx1+ neurons, a population of postmitotic V1 interneurons that express the transcription factor paired box gene 2 (pax2) (Burrill et al., 1997) is present in the unlesioned spinal cord. The number of pax2+ interneurons is unchanged at 2wpl or 6wpl. However, 17% of pax2+ interneurons at 6wpl co-label

with BrdU despite unchanged cell numbers after the lesion. This suggests that damaged pax2+ interneurons are replaced by new ones (Kuscha et al., 2012). Parvalbuminergic (parvalbumin7+) cells located in the dorsal horn of the spinal cord are rarely newly generated after a lesion (Kuscha et al., 2012). Differences in the lesion-induced neurogenic response of different classes of spinal neurons are apparent.

It is as yet unclear whether regeneration of spinal neurons contributes to functional recovery (Reimer et al., 2009). However, it is now well established that the recognition molecule L1.1, successful and spontaneous regrowth of axons in descending tracts rather than ascending tracts, the ability of axons to navigate novel pathways in the spinal gray matter and reorganization of the intra-spinal circuitry contribute to recovery of locomotor function after a spinal cord injury (Becker et al., 1997; Becker et al., 2004; Becker et al., 2005; Kuscha et al., 2011).

3.1.3 Role of Notch signalling in the lesioned spinal cord

The spinal cord of adult zebrafish is not a constitutively active neurogenic zone (Reimer et al., 2008), similar to mammals (Yamamoto et al., 2001; Meletis et al., 2008). However as mentioned above, in contrast to mammals, high numbers of neurons are generated from endogenous progenitor cells after a lesion (Reimer et al., 2008; Kuscha et al., 2011). Progenitor cells in the spinal cord of adult mammals are triggered to proliferate by a lesion, but these cells only generate glial cells (Meletis et al., 2008). However, they can generate neurons in vitro or when transplanted into neurogenic regions of the CNS (Shihabuddin et al., 2000).

In the mammalian spinal cord, activation of the Notch signaling pathway has been proposed to be responsible for the inability of spinal progenitors to generate neurons (Yamamoto et al., 2001). In several developing and adult systems, contact activation of Notch receptors on progenitor cells by Delta or Jagged ligands, present on differentiating progeny or newly recruited progenitors, inhibits

proliferation and/or neurogenesis from progenitor cells, thus providing a negative feedback mechanism for their own generation (recently reviewed in Ables et al., 2011; Pierfelice et al., 2011). Indeed, *notch1* expression is increased in the lesioned spinal cord of rats and activation of Notch inhibited, whereas attenuation of Notch signaling increased neurogenesis from spinal progenitors in vitro (Yamamoto et al., 2001). Therefore, we asked whether Notch pathway activation also occurs during successful neuronal regeneration in the lesioned spinal cord of adult zebrafish and whether it can be manipulated to generate more motor neurons. This cell type is of major therapeutic interest, because motor neurons are permanently lost after spinal injury and in motor neuron disease in humans.

Here we show that increased Notch signaling plays an attenuating role in motor neuron regeneration in the adult zebrafish spinal cord, consistent with a feedback model of Notch activation in progenitor cells. Remarkably, pharmacological inhibition of Notch signaling even increases generation of motor neurons in this adult vertebrate, which may have future therapeutic implications.

3.2 Results

3.2.1 The Notch pathway is activated in the lesioned spinal cord

Progenitor cells in the zebrafish spinal cord are located in the ependymal zone around the central canal (Reimer et al., 2008). To determine whether the Notch pathway is active in the lesioned spinal cord, we used in situ hybridization of the *hairy-related* (*her*) genes, many of which are direct Notch target genes (Chapouton et al., 2011), its ligands and receptors. The synthesized mRNA probes for in situ hybridization were validated initially in 24hpf wild-type embryos (n = 10 for each gene). The *Hairy-related* (*her*) genes such as *her4.1, her4.5, her8a, her13* and *hes6* were widely expressed throughout the embryonic CNS including spinal cord neurons. Similarly, the Notch receptors *notch1a*, *notch1b* and ligands *deltaA*, *deltaD*, *jagged1a* and *jagged2* were also strongly expressed in the brain and spinal

cord. However, some genes were not explicitly expressed in the CNS such as *her9*, which was expressed in hindbrain neurons and tail somites. *Notch2* was expressed in tail somites and *jagged1b* in hindbrain neurons and tail somites. *DeltaB* was expressed in the hindbrain and caudal spinal cord whereas *deltaC* was expressed in the telencephalon and somites. The observed in situ patterns resembled and were in accordance with previously published results (Fig. 3.1). This confirmed the accuracy and quality of the mRNA probes.

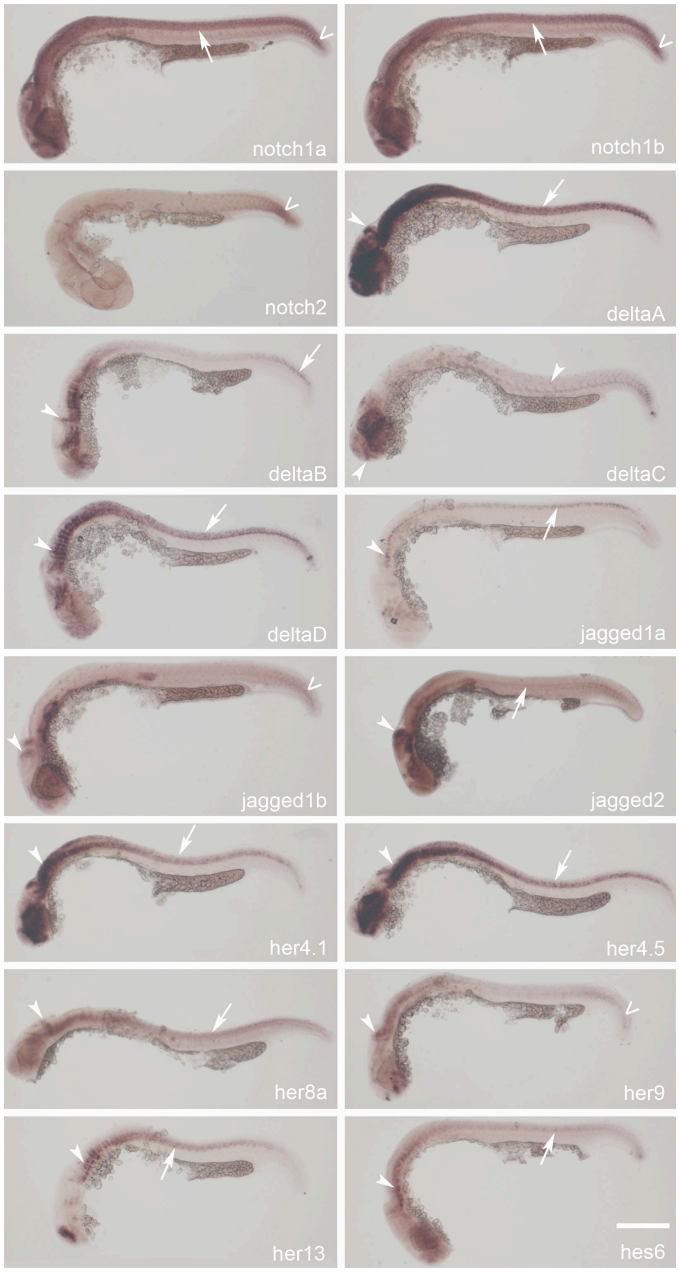

Fig. 3.1. mRNA probes for Notch genes are validated using whole mount in situ hybridization. In situ hybridizations in 24hpf wild-type embryos are shown here; arrowheads denote expression in hindbrain neurons, arrows mark expression in spinal cord neurons and open arrowheads indicate expression in somites in the embryonic tail. Notch receptors - 1a, 1b, ligands - *deltaA*, *deltaD*, *jagged1a*, *jagged2 and hairy-related* (*her*) genes - *her4.1, her4.5, her8a, her13, hes6* were strongly expressed in the embryonic brain and the spinal cord. In contrast, *her9, notch2* and *jagged1b* were expressed in hindbrain neurons and tail somites. Neurons in the hindbrain and caudal spinal cord expressed the ligand *deltaB* whereas neurons in the telencephalon and the somites in the tail expressed *deltaC*. Scale bar = 400μm.

Proliferation of spinal progenitor cells and motor neuron regeneration peaks at 14 dpl in the lesioned spinal cord, returning to unlesioned levels at 6wpl (Reimer et al., 2008). Inhibiting sonic hedgehog signalling during the regenerative period at 3, 6 and 9 dpl with an antagonist cyclopamine, reduced the number of motor neurons by 50% (Reimer et al., 2009). Similarly, to assess whether Notch signalling is functional and necessary for motor neuron regeneration in the lesioned spinal cord we analysed all data during this regenerative period and at 14dpl. We thus analyzed the expression of *her4.1*, *her4.5, her8a, her9, her13*, and *hes6* at 14 dpl and in the unlesioned spinal cord (n = 3 for each gene). *Her4.1* was upregulated at 3, 7 and 14 dpl from undetectable levels predominantly in a ventro-lateral domain of the ventricular zone (n = 3; Fig. 3.2; 3.3 A, B). No signal was present in the ventral midline zone, occupied by *sonic hedgehog* expressing ependymo-radial glial cells (Reimer et al., 2009). *Her4.5* was upregulated from undetectable levels in scattered cells around the central canal. *Her9* was expressed in dorsal midline cells in the unlesioned spinal cord and this expression domain expanded to comprise the dorsal half of the ventricular zone in the lesioned spinal cord (n = 3; Fig. 3.3 A, B). None of the other *her* and *hes* genes were detectably expressed in the unlesioned or lesioned spinal cord.

The receptors *notch1a* and *notch1b* were similarly upregulated from undetectable levels around the central canal, excluding the very dorsal and ventral domain. *Notch2* was upregulated from undetectable levels to a faint signal around the central canal (n = 3; Fig. 3.3 C).

The notch ligand *jagged1b* was upregulated from undetectable levels in ventricular cells of the dorsal midline and the ligand *deltaC* was upregulated in cells in the vicinity of the ventricular zone, predominantly in the ventral half of the spinal cord (n = 3; Fig. 3.3 D). None of the Notch ligands *deltaA*, *deltaB*, *deltaD*, *dll-4*, *jagged1a,* and *jagged2* were detectably expressed in the unlesioned and lesioned spinal cord. Thus Notch pathway genes are upregulated after spinal lesion in

specific dorso-ventral domains around the ventricle, probably reflecting the dorso-ventral polarity of the adult progenitor domains (Reimer et al., 2009).

Next we tested expression of *her4.1 and notch1b* in motor neuron progenitor cells in olig2:GFP transgenic animals. In single optical sections, in situ hybridization signals for both genes coincided with GFP labeling of olig2:GFP^{+} ependymo-radial glial cells (n = 3; Fig. 3.3 E), which are motor neuron progenitor cells (Reimer et al., 2008).

In a HB9:GFP transgenic fish, expression of *deltaC* mRNA overlapped with small, strongly GFP expressing, newly-generated motor neurons (Reimer et al., 2008) (n = 3; Fig. 3.3 E). Thus Notch signaling is probably activated in motor neuron progenitor cells after a spinal lesion, as indicated by upregulation of the target gene *her4.1*. Receptor (*notch1b*) expression is upregulated in the same cell type and a ligand (*deltaC)* is expressed by the motor neuron progeny of these cells.

It is important to note that co-labelling between the in situ signal and the GFP immunofluorescence was identified in single optical sections nevertheless these results are a qualitative assessment. It would be beneficial to back-up these observations with a quantitative analysis of the co-labelling events. However, this technique suffers from the limitation that the strong in situ signal can quench the GFP fluorescence making it difficult to quantify the number of co-labelled cells. In addition, the bound mRNA is localized in the cytoplasm of the cell similar to the location of GFP transgene expression making it difficult to delineate one cell from the neighboring cell. An alternative approach to overcome this limitation could have been the use of fluorescent in situ hybridization with immunohistochemistry for GFP together with a DAPI counterstain. With the use of a fluorescent dye to label the mRNA of interest we would get rid of the quenching effect and with the DAPI counterstain it would be possible to clearly demarcate the cells that are co-labelled.

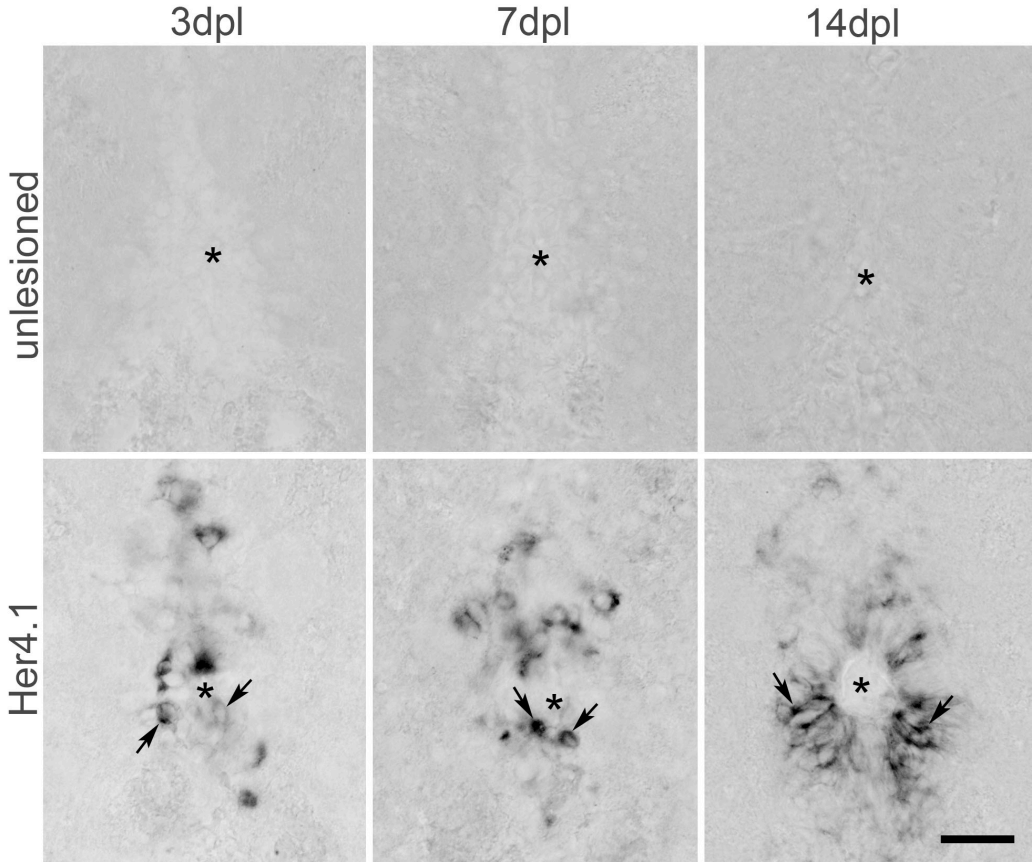

Fig. 3.2. Downstream target gene of Notch, *hairy-related* (*her*) *4.1* or *her4.1* is upregulated after a spinal lesion. In situ hybridizations for *her4.1* in crossections of the unlesioned and lesioned spinal cord at shown; asterisks mark the central canal, dorsal is up and arrows indicate *her4.1* mRNA expression. *Her4.1* is upregulated in ependymo-radial glial cells at 3, 7 and 9 days post lesion from undetectable levels in the unlesioned spinal cord. Scale bar = 50μm.

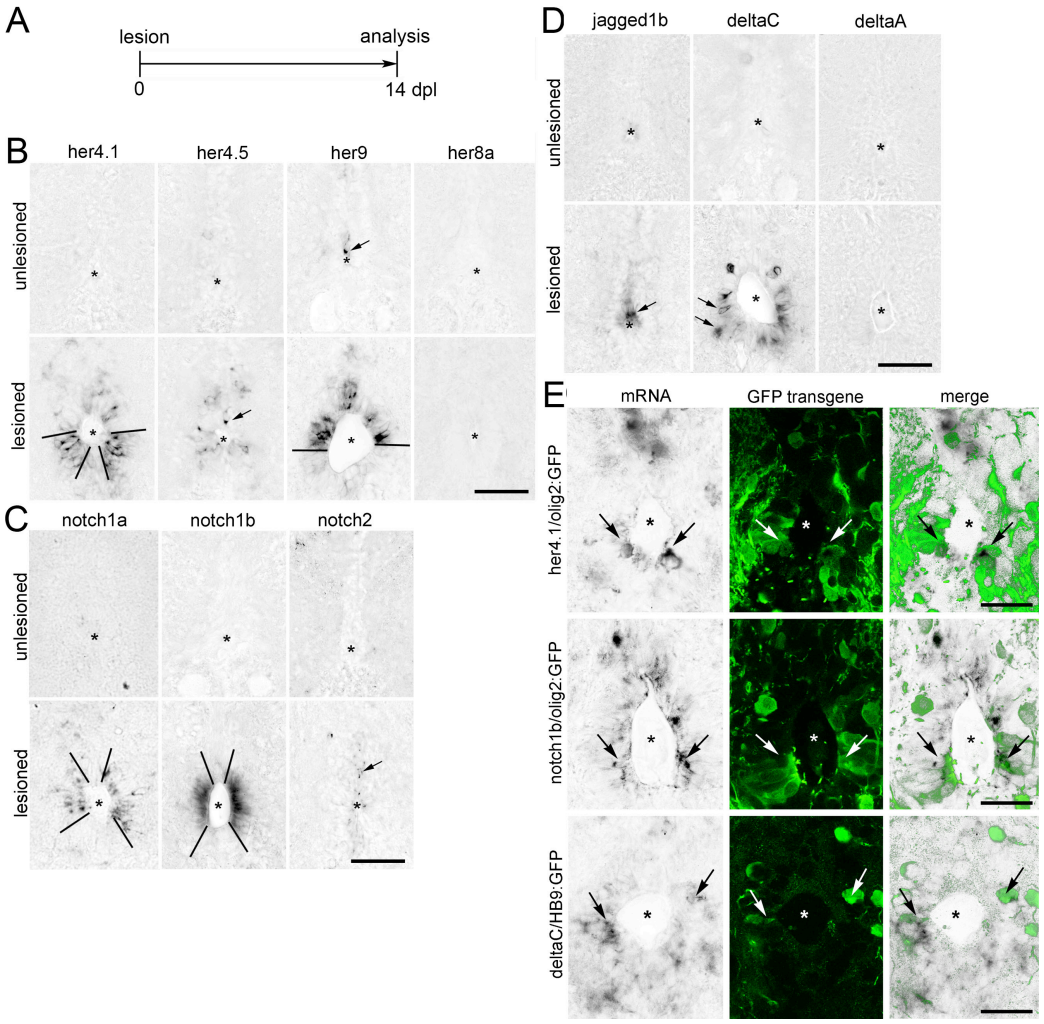

Fig. 3.3. Notch pathway genes are upregulated after spinal injury. In situ hybridizations of spinal cross sections at 14 dpl are shown; central canal is indicated by asterisks; dorsal is up; in B-D arrows indicate single positive cells and bars delineate expression domains. A: Timeline for all experiments in B-E. B: After a lesion, her4.1, her4.5 and her9, but not her8, are markedly increased in expression in specific areas of the ependymal zone. C: After a lesion, notch1a and notch1b are strongly upregulated in specific regions of the ventricular zone, whereas notch2 shows weakly increased expression after a lesion. D: After a lesion, jagged1b is upregulated only in the dorsal midline, whereas deltaC is upregulated preferentially in cells around the ventral half of the central canal, including cells that are one cell diameter distant from the central canal. DeltaA is not detectably expressed after a lesion. E: In situ hybridizations and anti-GFP labeling of transgenes in single optical sections of spinal cross sections are shown. Arrows mark co-labeling of transgene and in situ hybridization signal. *Her4.1* and *notch1b* expression is found in olig2:GFP+ ventricular progenitor cells, whereas deltaC expression is found in HB9:GFP+ motor neurons. Scale bars = 25 µm.

3.2.2 Notch over-activation strongly inhibits generation of motor neurons and attenuates progenitor cell proliferation in the lesioned spinal cord

Double-transgenic fish, Tg(hsp70l:Gal4) x Tg(UAS:myc-notch1a-intra), in which a heat-shock promoter drives expression of the active intracellular domain of notch1a, fused to a myc epitope, allow conditional and potent over-activation of the pathway (Scheer et al., 2001). Myc immuno-reactivity was detected in the spinal cord of unlesioned and lesioned transgenic animals after heat-shocks on two consecutive days, but not in heat-shocked wildtype or non heat-shocked transgenic animals (n = 3; Fig. 3.4 A). Remarkably, immuno-reactivity was predominantly present in ependymo-radial glial cells, as indicated by labeling of radial processes of these cells, providing some specificity of the manipulation for these spinal progenitor cells. Myc-immunoreactivity was markedly stronger in the nucleus of the cells, indicating correct transport of the activated protein to the nucleus and specificity for ventricular cells was maintained in lesioned animals even after daily heat-shocks from 5-13 dpl (n = 3; Fig. 3.4 B). PCR of spinal tissue surrounding the lesion site indicated increased expression of her4.1 (Fig. 3.4 C). In unlesioned animals, 5 consecutive heat-shocks induced detectability of the target gene her4.1 around the central canal, except for the most ventral cells, by in situ hybridization (n = 3; Fig. 3.4 D), showing that Notch activation was functional. Thus, the heatshock and not a lesion activates Notch in spinal progenitor cells.

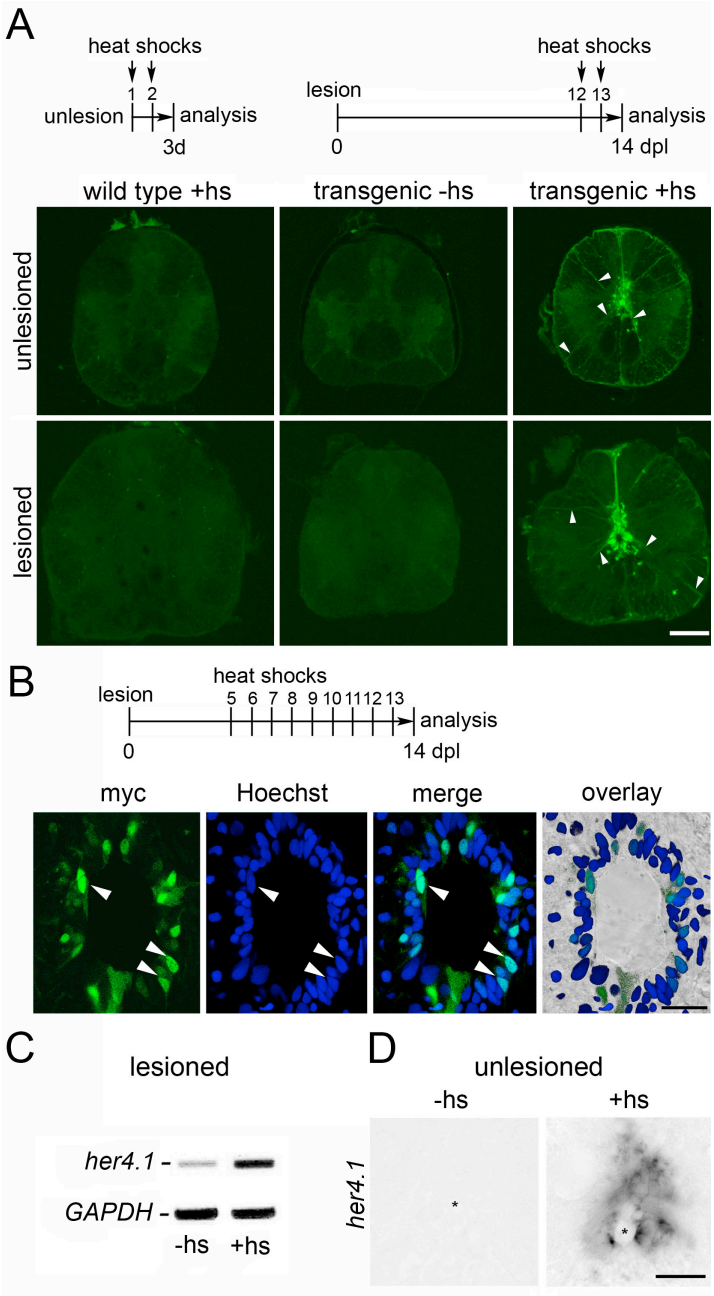

Fig. 3.4 Heat-shock induced over-activation of Notch in ependymo-radial glial cells of *Tg(hsp70l:Gal4) x Tg(UAS:myc-notch1a-intra)* transgenic fish. Spinal cross sections are shown. **A:** Ventricular myc immuno-reactivity is observed in ependymo-radial glial cells and their processes (arrowheads) in double transgenic animals after two heat-shocks (hs) in unlesioned and lesioned animals. **B:** At higher magnification of a single optical section heat-shocked at the indicated intervals, myc-immunoreactivity is increased in a subset of ependymal cells (see text for quantification). The signal is still seen mostly in ependymal cells and is strongest in the cells' nuclei, as revealed by comparison with nuclear counterstaining (arrowheads). **C:** *Her4.1* expression is increased in the spinal cord of

lesioned transgenic animals heat-shocked in the same way as in B, but analyzed at 10 dpl by PCR. **D:** In unlesioned animals, 5 daily heat-shocks elicit *her4.1* expression in the ependymal region as detected by in situ hybridization. Asterisks indicate the central canal. Scale bar in A = 50 µm, in B = 25 µm, in D = 25 µm.

To over-activate Notch signaling during motor neuron regeneration, daily heat-shocks were delivered from 5-13 dpl. This treatment induced detectability of myc-labeling in 29% of all ventricular cells (Hoechst labeling: 57.61 ± 2.67 cells/ optical section, myc double-labeled cells: 16.61 ± 3.53, n = 3 animals) at 14 dpl. In the postembryonic spinal cord, some ependymo-radial glial progenitors are refractory to alterations in Notch signalling (Kim et al., 2008). This phenomenon could be recapitulated in the lesioned spinal cord and might explain the patchy myc-labelling upon Notch activation. The myc negative progenitors probably require another locally active signalling pathway for subsequent cell fate decisions.

The number of small $HB9^+$ cells in the lesioned spinal cord was determined at 14 dpl. Previous BrdU labeling studies have indicated that such cells are newly generated motor neurons, which are rare in the unlesioned spinal cord (Reimer et al., 2008). In lesioned, non heat-shocked transgenic controls, clusters of intensely labeled small $HB9^+$ motor neurons were observed in the ventro-medial aspect of spinal cross sections (Fig. 3.5 A - C) in a number that was comparable to previous observations in wildtype animals (Reimer et al., 2008) and not statistically different from that observed in heat-shocked wildtype animals at 14 dpl (Fig. 3.5 A - C). This supported that the presence of the transgenes or heat-shocks per se did not influence motor neuron generation. However, in heat-shocked transgenic animals, the number of small $HB9^+$ cells was dramatically reduced by 87% in the 1500 µm surrounding the lesion site (Fig. 3.5 A - C).

To test whether a lack of motor neuron generation was at least in part due to inhibition of ventricular proliferation we used PCNA immunohistochemistry, which labels cells in early G1 phase and S phase of the cell cycle. Proliferating

cells were mostly found in the ependymal zone around the central canal, including the motor neuron progenitor domain, as previously reported (Reimer et al., 2008). The overall number of $PCNA^+$ cells in the ependymal zone within 1500 μm surrounding the lesion site was reduced by 46%, after heat-shocks (Fig. 3.5 A, D, E), indicating that Notch activation inhibits progenitor cell proliferation.

At the cellular level we found that in non heat-shocked double-transgenic animals 25% of all ventricular cell profiles were $PCNA^+$ (Hoechst: 61.1 ± 4.96 cells/ optical section; PCNA double-labeled: 15.22 ± 4.12, n = 4), similar to wild type controls (25%; Hoechst: 77.52 ± 9.85 cells/optical section, n = 3; PCNA: 19.56 ± 3.23, n = 3) at 14 dpl. In heat-shocked double-transgenic animals, this rate was reduced to 10% (Hoechst: 57.46± 2.27 cells/ optical section; PCNA double-labeled cells: 5.85 ± 1.18; n = 9), confirming stereological counts. If activation of Notch inhibits cell proliferation, PCNA labeling should be reduced in the same cells that over-express active Notch. Indeed, only 2.7% of all conspicuously myc-labeled cells were labeled with PCNA antibodies. This was highly significantly less than the expected value of 25% (the overall proportion of ventricular cells that are labeled by PCNA antibodies in non-heat-shocked controls). The remaining $PCNA^+$ cells in heat-shocked animals intercalated with myc-labeled cells (Fig. 3.5 F). This provides direct evidence that Notch activation in progenitor cells reduces their proliferative activity. Thus, the heat-shock drives over-activation of Notch signalling in responsive proliferating spinal progenitors which in turn strongly inhibits subsequent generation of new motor neurons.

The proliferation of spinal progenitors and the subsequent generation of new motor neurons peaks at 2wpl in the lesioned spinal cord (Reimer et al., 2008). Consequently, this strongly suggests that the decrease in PCNA counts observed after activation of Notch signalling is a primary consequence of the over-expression. However, it is important to note that without a time course study it is difficult to exclude the possibility that the effect on PCNA counts is not a

secondary consequence of Notch activation. There are two possible interpretations to favor this scenario. Firstly, overexpression of Notch activity could affect proliferation and subsequent cell fate decisions over time. Secondly, Notch could promote another neural fate while inhibiting the motor neuron fate thus reducing the overall number of PCNA positive profiles. Indeed, changes in Notch activity are instrumental in regulating binary cell fate choices in vertebrates, leading to multiple neuronal subtypes (Cau and Blader, 2009).

Inhibitory GABAergic interneurons (KA') and primary motor neurons are produced simultaneously in the pMN domain in the developing zebrafish spinal cord where one KA' and one motor neuron cell are generated from a terminal asymmetric division. Gain-of-function studies show that constitutive Notch activity promotes the specification of the KA' fate over a motor neuron fate (Shin et al., 2007)(Park et al., 2004). The number of V2 interneurons that arise from the p2 progenitor domain adjacent to the pMN domain is increased 24-fold at 2wpl in the adult lesioned spinal cord (Kuscha et al., 2012). Notch has been shown to influence the specification of V2 interneurons from the p2 domain in the murine and zebrafish spinal cord (Peng et al., 2007) (Batista et al., 2008; Kimura et al., 2008).

Oligodendrocytes myelinate axons in the CNS and arise from progenitors in the pMN domain that earlier produced motor neurons. Over-activation of Notch signalling has been shown to promote the specification of oligodendrocyte precursors at the expense of motor neurons (Park and Appel, 2003). It is possible that following Notch activation in the lesioned spinal cord, spinal progenitor cells differentiate into interneurons (KA' or V2) or oligodendrocyte precursors while inhibiting motor neuron generation. In this thesis, I have not addressed the effect of constitutive Notch activation on interneurons or oligodendrocyte precursors but it would be informative for future studies.

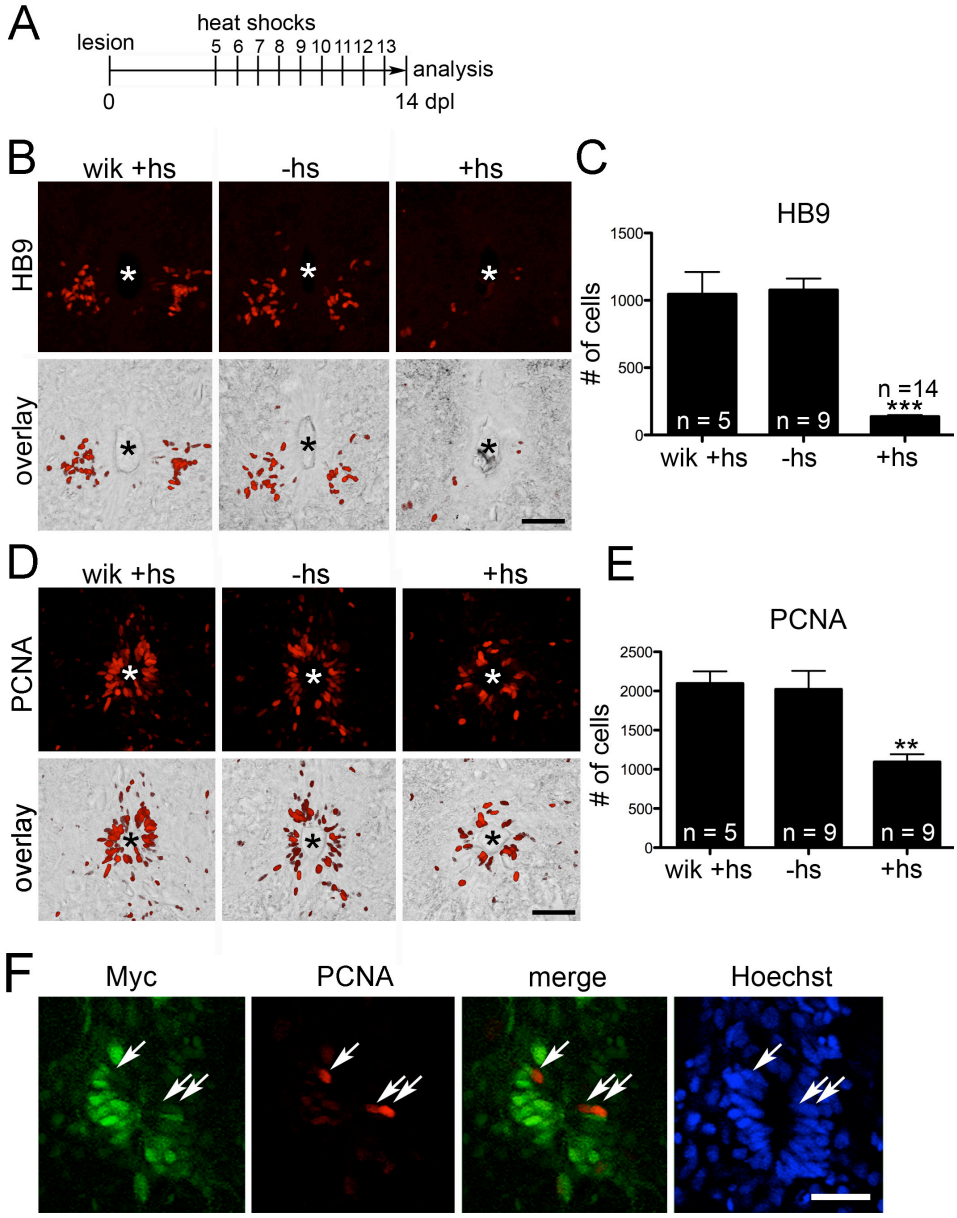

Fig. 3.5 Forced activation of Notch signaling strongly reduces generation of new motor neurons and proliferation in the lesioned spinal cord. Spinal cross sections, centered around the central canal (asterisks in B,D), are shown; dorsal is up. **A:** The experimental paradigm used in B-F is shown. **B,C:** The number of $HB9^{+}$ motor neurons in the ventro-medial aspect of the spinal cord is strongly (***$P < 0.001$) reduced. **D, E:** The number of periventricular $PCNA^{+}$ cells is likewise reduced by heat-shock treatment. Overlay indicates immuno-reactivity superimposed on phase-contrast images (**$P < 0.01$). **F:** Myc and PCNA immuno-reactivities are mutually exclusive in the ependymal layer. Arrows indicate the position of strongly PCNA immuno-reactive cells, which are not labeled by anti-myc antibodies. Hoechst labeling indicates the outline of the ependymal layer. Scale bars = 50 µm.

3.2.3 Inhibiting Notch activity increases the number of newly-generated motor neurons and progenitor cell proliferation in the lesioned spinal cord

The gamma-secretase inhibitor DAPT prevents cleavage of the Notch receptor into its active form and has been shown to attenuate Notch signaling in adult zebrafish (Chapouton et al., 2010). Indeed a single intraperitoneal injection at 4 dpl was sufficient to reduce detectability of *her4.1* in PCRs of lesioned spinal tissue at 5 dpl (Fig. 3.6 A), indicating successful manipulation with this procedure of Notch signaling in ventral spinal progenitor cells, in which *her4.1* is predominantly expressed.

Injections of DAPT (3, 6, 9 dpl) during the regeneration period strongly increased the number of newly generated $HB9^{+}$ motor neurons by 61% and that of proliferating $PCNA^{+}$ ventricular cells by 37% at 14 dpl (Fig. 3.6 B-F). However, in the adult viable *notch1a* mutant, numbers of newly generated motor neurons appeared unaffected at 14 dpl (non-homozygous siblings: 1244 ± 114.1, n = 10 animals; mutants: 1050 ± 172.5, n = 8 animals, $P > 0.05$), possibly due to compensation by *notch1b*. Thus chemically inhibiting Notch signaling augments progenitor proliferation and motor neuron generation.

As we show here for the first time that the number of newly generated motor neurons can be augmented using DAPT, we wanted to ascertain that the drug had no detrimental effects on subsequent recovery. These could be caused by destroying the ependymal zone as a consequence of progenitor depletion (Carlén et al., 2009) or by off-target toxicity. In a test in which the swim endurance of fish is measured in a flow of water and in an assessment of the total distance moved within 5 minutes (Reimer et al., 2009), DAPT-injected animals significantly recovered in both tests between 3 and 6 weeks post-lesion. DAPT-treated animals performed slightly better than controls, but this difference was not significant (Fig.

3.6 G,H). This indicates that DAPT has no overt toxic effect on regenerating fish and that increased numbers of HB9^{+} motor neurons at 2 weeks post-lesion do not negatively impact subsequent functional recovery.

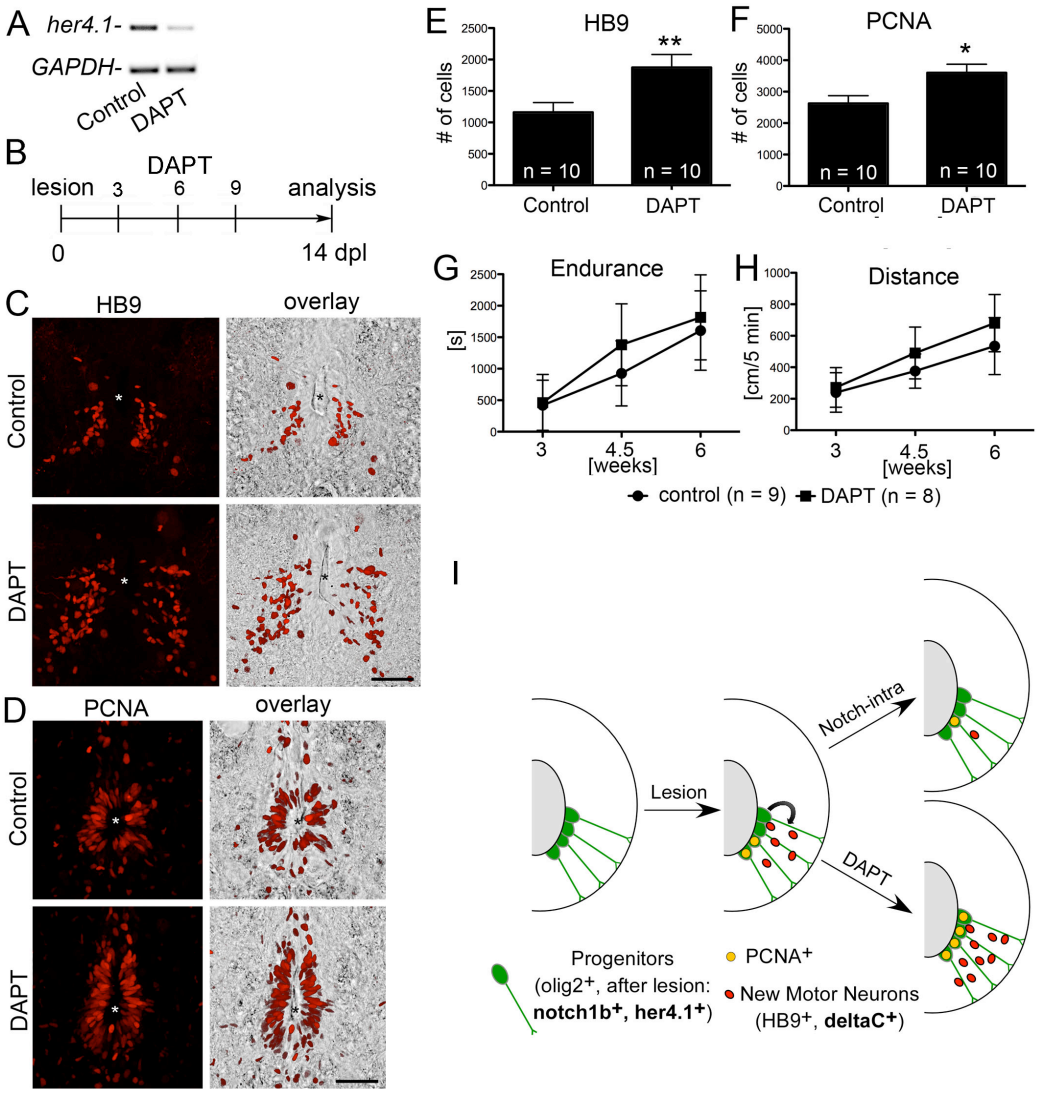

Fig. 3.6 DAPT treatment increases motor neuron generation and ventricular proliferation and does not impair recovery. Part of spinal cross sections, centered around the central canal (asterisks), are shown in B,C; dorsal is up. **A:** A single injection of DAPT reduces detectability of *her4.1* expression in PCR of the lesioned spinal cord. *GAPDH* levels were similar in the treated and untreated spinal cord. **B:** The treatment and analysis regimen used in C-F is indicated. **C, E:** DAPT treatment significantly increases the number of HB9 immuno-reactive newly generated motor neurons in the ventro-medial aspect of the spinal

cord (**P < 0.01). **D, F:** $PCNA^+$ cells are significantly increased in number (*P < 0.05) around the central canal in DAPT-treated animals at 14 dpl. **G, H:** Endurance in a flow (G; P < 0.05) and the total distance moved (H; P < 0.01) similarly improved over time for DAPT-treated (as in B but with the indicated time points of analysis) fish, and were not significantly different from vehicle-injected controls. **H:** Schematic summary of results. Over-expression of the intracellular domain of Notch1a attenuates, whereas DAPT treatment augments lesion-induced progenitor cell proliferation ($PCNA^+$) and generation of motor neurons. Scale bars: 50 µm.

3.2.4 Notch manipulations have no detectable effect on progenitor cell activity in the unlesioned spinal cord

In the unlesioned spinal cord motor neuron generation and ventricular proliferation is rare. Over-activation of Notch by 9 daily heat-shocks (analysis on day 10) did not lead to any changes in the low numbers of small HB9+ motor neurons (non-heat-shocked: 70.61 ± 12.11 cells/1500 µm spinal cord, n = 3; heat-shocked: 50.19 ± 7.43, n = 4; P > 0.05) or PCNA+ cells at the central canal (non-heat-shocked: 64.29 ± 22.96 cells/1500 µm spinal cord, n = 3; heat-shocked: 65.95 ± 19.51, n = 4; P > 0.05). Similarly, daily DAPT injections for 5 consecutive days (analysis on day 8) had no influence on the low numbers of small HB9+ motor neurons (DMSO treated: 137.49 ± 18.13 cells/1500 µm spinal cord, n = 12; DAPT treated: 156.72 ± 13.89, n = 10; P < 0.05) or PCNA+ cells (DMSO treated: 41.35 ± 5.31 cells/1500 µm spinal cord, n = 12; DAPT treated: 40.31 ± 5.94, n = 10; P < 0.05) at the central canal. This shows that additional signals from the lesion are needed to make spinal progenitor cells competent to react to manipulations of Notch signaling.

3.3 Discussion

We show here, that lesion-induced Notch signaling attenuates proliferation of progenitor cells and motor neuron generation from these cells in the lesioned spinal

cord of adult zebrafish. We also demonstrate for the first time that motor neuron generation can be augmented in this adult vertebrate by pharmacologically blocking Notch signaling (summarized in Fig. 3.6 H).

Manipulations of Notch activity after a spinal lesion in zebrafish indicate that Notch is a negative regulator of both proliferation in the ventricular progenitor cell zone and of motor neuron generation. This is similar to the role of Notch during constitutive neurogenesis in the forebrain of zebrafish (Chapouton et al., 2010; Rothenaigner et al., 2011) and is compatible with a role of Notch signaling in maintaining progenitor pools. Remarkably, the function of Notch in the regenerating spinal cord of zebrafish is similar to its proposed role in the lesioned spinal cord of rats, where Notch is strongly upregulated and activated Notch acts as an anti-neurogenic factor in vitro (Yamamoto et al., 2001). It is possible that expression levels of active Notch are too high to allow neurogenesis in the lesioned mammalian spinal cord. As we show here, expression of the active form of Notch by the strong Gal4/UAS activator system in addition to endogenous Notch activity dramatically reduced the number of newly-generated motor neurons also in the spinal cord of adult zebrafish.

Conversely, in the adult mouse telencephalon, where stroke induces neuroblast formation from ependymal cells, Notch signaling is decreased and ependymal cells are eventually consumed by this differentiation process. Ependymal cells can be rescued by activating Notch (Carlén et al., 2009). However, when we reduced Notch signaling with DAPT, we did not observe any obvious disruption of the spinal ependymal zone and DAPT treatment did not negatively affect recovery of swimming function. Such disruption might occur with stronger or longer inhibition of Notch. Overall, our results best support the hypothesis that precise regulation of Notch activity is necessary to allow neurogenesis, while maintaining the progenitor pool in the lesioned CNS.

In the unlesioned spinal cord there was little evidence of neurogenesis (Reimer et al., 2008) and Notch activity. For instance, *her4.1* expression, which is positively regulated by Notch signaling (Chapouton et al., 2011), and expression of Notch receptors was undetectable. Similarly, DAPT had no effect on proliferation and motor neuron generation. Even when the active form of Notch was over-expressed, leading to increased *her4.1*expression in the ependymal zone, proliferation and motor neuron generation were not influenced. This contrasts with progenitor zones in the adult brain of zebrafish and mice that show constant activity of Notch signaling (Carlén et al., 2009; Chapouton et al., 2010). These observations suggest that ependymo-radial glial cells in the adult zebrafish spinal cord need an unknown signal from the lesion event that switches on Notch signaling and leads to neurogenesis (Reimer et al., 2008; Reimer et al., 2009; Kuscha et al., 2011). This supports an association of Notch signaling with neurogenesis.

Interestingly, the re-activation of Notch signaling is not an exact recapitulation of developmental gene expression. For example, *deltaA, deltaD, jagged1a*, which are strongly expressed in the embryonic spinal cord, and *jagged2*, which is expressed in embryonic motor neurons (Yeo and Chitnis, 2007), are not detectably expressed in the adult lesioned spinal cord. Vice versa, *deltaC* is upregulated in the lesioned adult spinal cord, but is hardly detectable in the embryonic spinal cord. Other genes, such as *notch1a*, *notch1b*, *her4.1* and *her4.5* are similarly expressed in development and regeneration (data for embryonic expression not shown and zfin.org).

How does Notch act? We observed that proliferative activity in the ependymal zone was reduced upon over-activation of Notch and increased when Notch signaling was blocked. This could have resulted in the differences in motor neuron numbers in a mechanism that is similar to that involved in neurogenesis in the constitutively active proliferation zones in the zebrafish forebrain. In that system, a lineage tracing study indicated that manipulations of Notch signaling only

influenced the proliferation rate of progenitor cells and not the type of division (symmetrical vs. asymmetrical) or cell differentiation (Chapouton et al., 2011). However, we cannot exclude that Notch has additional roles in cell fate decisions of neuroblasts during spinal cord regeneration, similar to development (Shin et al., 2007; Yeo and Chitnis, 2007; Batista et al., 2008; Kimura et al., 2008). In the proliferative zones of the adult mammalian forebrain, Notch signaling either negatively (Imayoshi et al., 2010) or positively (Androutsellis-Theotokis et al., 2006) regulates proliferation, possibly depending on the progenitor cell type analyzed (Pierfelice et al., 2011).

Increased numbers of motor neurons after DAPT treatment did not improve recovery of fish from the spinal lesion. This is perhaps not surprising, as we have shown previously that less than 20% of the newly-generated HB9 expressing motor neurons fully differentiate into choline acetyl transferase positive motor neurons (Reimer et al., 2008) and reducing the number of newly-generated motor neurons by 50% also did not affect recovery (Reimer et al., 2009). Thus the potential beneficial effect of new neurons may be limited by the number of neurons that can be integrated into the system. However, this situation might be different in mammals, in which hardly any neuroblasts are generated in the adult lesioned spinal cord (Shihabuddin et al., 2000; Yamamoto et al., 2001; Meletis et al., 2008).

We conclude that Notch signaling is precisely regulated to allow motor neuron regeneration in the lesioned spinal cord of adult zebrafish. Pharmacological intervention with the Notch inhibitor DAPT increases motor neuron generation from endogenous stem cells, which may inform future therapeutic interventions in the lesioned spinal cord of mammals in which Notch signaling is strong and neurogenesis does not occur (Shihabuddin et al., 2000; Yamamoto et al., 2001; Meletis et al., 2008).

4. The role of notch in neurogenesis in the intact adult zebrafish retina

4.1 Introduction

In the lesioned spinal cord of adult zebrafish, I find that activating the Notch signalling pathway attenuates motor neuron generation and subsequent proliferation. However, in the unlesioned retina, the same manipulation led to hyperproliferation of cells in the inner nuclear layer (INL). This incidental find may provide the first hint that the role of Notch may differ in different adult progenitor cell pools and will lead to future investigations of Notch-induced neurogenesis in the retina.

4.1.1 Anatomy of the zebrafish retina

The vertebrate retina is derived from the neural plate, as it is part of the central nervous system. It is remarkable that the distinctive structure of the retina is highly conserved across species ranging from primates to teleost fish. A histological view of the neural retina reveals a regular pattern of cells arranged in different layers (Fig 4.1). The three main cellular layers in the retina are the outer nuclear layer or ONL, the inner nuclear layer or INL and the ganglion cell layer or GCL. The retina consists of six major classes of neurons and one glial cell type. The elongated cell bodies of the photoreceptors that include cones and rods are radially arranged to form the outermost layer of the retina or the ONL. The cone photoreceptors are involved in the perception of bright light and colour vision. The rod photoreceptors on the other hand respond to low levels of light and their sensitivity is higher than that of the cones.

The innermost layer of the retina, i.e. the GCL, is made up of ganglion neurons, or projection neurons. Three classes of interneurons, namely bipolar cells, horizontal cells and amacrine cells constitute the innermost layer of the retina or the INL. The interneurons in the INL are also found to be stratified. The horizontal cells are arranged tangentially in the outer level of the INL whereas the amacrine cells occupy the inner row of the INL. The bipolar cells are located in the vicinity in

between the horizontal and amacrine cells. The cell bodies of Müller glia lie in the INL with their processes spanning all the three cellular layers. The photoreceptors, bipolar and horizontal cells make synaptic connections in the outer plexiform layer. The bipolar cells, amacrine cells and ganglion neurons communicate with each other in the inner plexiform layer (Dowling, 1970; Dowling and Werblin, 1971).

Visual phototransduction is mediated by G-protein coupled receptors called opsins bound to the cell membranes of photoreceptors. The opsins contain a chromophore called 11-cis-retinal. On stimulation, the flow of visual information is vertically transferred from the opsins in the photoreceptors via bipolar cells in the INL to the ganglion neurons in the GCL. The horizontal cells and amacrine cells in the outer and inner plexiform layers also transmit the information laterally and thus adapt the direct signalling from the photoreceptors to the ganglion cells. These signals then travel from the GCL along the optic nerve to the brain (see Fig 4.1).

4.1.2 Cellular diversity in the retina

The defined architecture of the retina with seven different cell types whose cell bodies and synaptic connections are arranged in specified layers is a simplistic view of a highly organized and complex structure. In likeness to the central nervous system, there is a huge neuronal diversity. In the mammalian retina there are approximately 55 distinct cell types (Masland, 2001). Zebrafish are diurnal and their retinas contain diverse cone subtypes in addition to the rod photoreceptors. Cones have been subdivided into four classes on the basis of differences in morphology and spectral sensitivity – short single cones (UV), long single cones (blue) and the red and green members of the double cone pairs (Raymond et al., 1993; Robinson et al., 1993; Raymond et al., 1995; Pujic and Malicki, 2004). There are four subtypes of horizontal cells (Song et al., 2008), seven subtypes of amacrine cells (Marc and Cameron, 2001), and eleven subtypes of ganglion cells (Mangrum et al., 2002) classified on the basis of morphology.

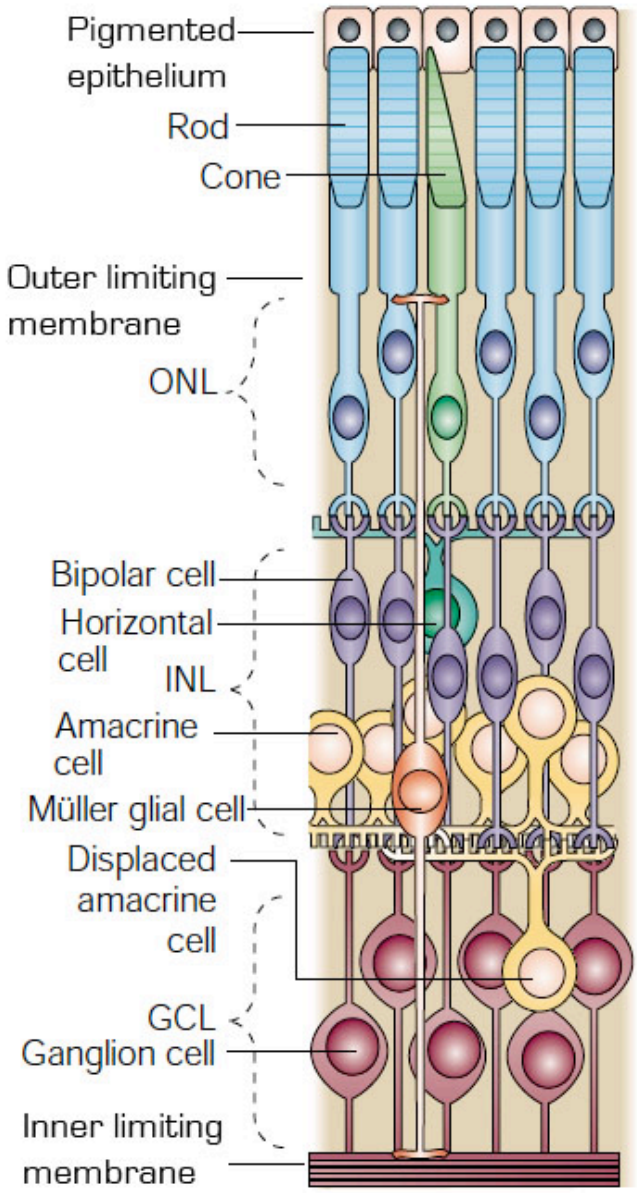

Fig 4.1. A histological view of the retina. Radially arranged nuclei of the rods (blue) and cones (green) make up the outer nuclear layer or ONL. The inner nuclear layer or INL contains horizontal cells (green), bipolar cells (purple), amacrine cells (yellow) and Müller glia (orange). The radial processes of the Müller glia contact the inner and outer limiting membranes. The ganglion cell layer or GCL primarily consists of ganglion neurons (red) and occasionally displaced amacrine cells. modified from (Dyer and Cepko, 2001).

4.1.3 Development of the retina

Development of the retina in all vertebrates begins from a uniform population of neuroepithelial cells in the anterior neuroectoderm in a stereotyped spatiotemporal pattern. The pattern of neurogenesis in the teleost retina is striking because its predictability has served as a useful tool to study the mechanisms and identification of factors essential to this process. The steps that are part of this developmental pattern are the fan-shaped neurogenic waves, specific timing of cell cycle exit in progenitors, cell polarity and migration, and the generation of a mosaic pattern.

Initiation of neurogenesis - fan shaped neurogenic waves

Upon neural induction, an evolutionarily conserved programme involving complex interactions between patterning homeodomain (HD) proteins such as paired box protein PAX6 (Macdonald et al., 1995) retinal homeobox protein RX1(Mathers et al., 1997; Chuang and Raymond, 2001), sine oculis homeobox homologs SIX3 and SIX6 (Oliver et al., 1995; Seo et al., 1998) and visual system homeobox 2 (VSX2) (Passini et al., 1997; Livne-Bar et al., 2006)form a group of eye-field transcription factors that specify the anterior neural plate for eye development (Zuber et al., 2003). These factors subdivide the neural plate into distinct domains and confer retinal progenitors with distinct positional identities.

Neurogenesis in the zebrafish retina initiates asynchronously in a small and distinct patch located in the ventronasal region. A subsequent wave of cellular differentiation then advances from the patch and spreads dorsally to the ventral temporal retina (Schmitt and Dowling, 1996; Hu and Easter, 1999; Easter and Malicki, 2002). This wave of neurogenesis resembles the opening of a fan and is different to the centrifugal neurogenic gradient seen during retinal development in other vertebrates. Neurogenesis in each layer of the retina proceeds in the same pattern except during the production of rod photoreceptors, which have a ventral-

to-dorsal developmental gradient (Stenkamp, 2007). This asynchronous pattern of retinal development is similar to the posterior to anterior neurogenic wave seen in Drosophila.

Specific timing of cell cycle exit in progenitors

The retinal progenitors that exit the cell cycle first differentiate into ganglion cells at ~28 hours post fertilization (hpf) (Raymond et al., 1995). The order of neurogenesis proceeds from the inner to the outer most cell types (Hu and Easter, 1999). This order of neuronal differentiation is consistent with observations made in other species (Carter-Dawson and LaVail, 1979; Stiemke and Hollyfield, 1995; Harris, 1997). Neurons in the INL are postmitotic at ~ 48hpf and the Müller glia are the last cell type to differentiate at ~ 72hpf. Müller glial maturation and differentiation requires neural-glial connections (Linser and Moscona, 1979; Moscona and Linser, 1983) with the involvement of Delta – Notch signaling (Peterson et al., 2001; Bernardos et al., 2007).

Cell polarity and migration

Newborn neurons migrate from the apical surface to the final positions they will occupy in the developed retina. The significance of cell polarity during the development of the retina was identified in zebrafish lamination mutants where cell migration is impaired and the resulting retina has severe patterning defects (Malicki et al., 1996). In a normal retina, as progenitor cells proliferate, the nuclei migrate as they pass through the different phases of the cell cycle. During the S-phase, nuclei migrate towards the vitreal surface and to the apical surface in the M-phase. N-cadherin has been shown to play a role in the laminar patterning of the retina (Malicki et al., 2003).

Mosaic organisation of the retina

The distinct laminar organization of the retina and the crystalline-like mosaic organization of neuronal cells together relay accurate visual signals (Fadool, 2003).

The red/green double cones are arranged in parallel rows that alternate with rows of the blue and UV single cones (Larison and Bremiller, 1990). The arrangement of the rod photoreceptors is variable. The rods are arranged in rows that alternate with the arrays of the cone photoreceptors (Fadool, 2003). Raymond et al., (1995) propose a model whereby lateral inductive interactions between differentiating photoreceptors regulates the formation of this highly ordered mosaic in the retina. Furthermore, it has been shown at the molecular level that inactivation of Delta - Notch signalling led to a disruption in the planar and laminar organization of all retinal neurons (Bernardos et al., 2005).

4.1.4 Regulation of cell fate

The progression from multipotent retinal progenitor cells to mature retinal neurons has been the subject of a longstanding debate in vertebrate retinal neurogenesis. How is this process regulated spatially and temporally? Initially, it was thought that the ability of uncommitted progenitors to generate different subtypes of postmitotic neurons decreases over developmental time (Reh and Cagan, 1994). However, a more comprehensive explanation came from the “competence” model of retinal development (Cepko et al., 1996). The model proposed that retinal progenitors move from one competence state to the next and these changes in competence generate different neurons.

Within each competence state, intrinsic regulatory mechanisms and positive/negative environmental or extrinsic factors in the progenitors that change over time regulate cell fate by conferring a given competence (Cepko, 1999) (Livesey and Cepko, 2001). Transcription factors initiate specific transcriptional programmes intrinsic to the progenitor cells, allowing responsiveness to extrinsic signals. Extrinsic factors regulate the final choice in cell fate and promote differentiation. The model described above was derived from studies in mice and chick, however studies in zebrafish have contributed to a better understanding of the regulation of the intrinsic and extrinsic cues.

4.1.5 Intrinsic factors

Progenitor maintenance

During the early stages of retinal development, inhibitory basic-helix-loop-helix (bHLH) Hes genes, homologues of the Drosophila hairy and Enhancer of split (Sasai et al., 1992) regulate proliferation of retinal progenitors in mammals. These Her genes act as prepattern factors in inter-neuronal domains during specification of the neuroectoderm and in the proneural domain in the midbrain-hindbrain boundary in zebrafish (Geling et al., 2004; Bae et al., 2005). Her/Hes genes are downstream targets of Notch signalling and thus play a role in the maintenance of a progenitor character (Gaiano et al., 2000) without affecting temporal identities (Jadhav et al., 2006). Another important factor required early in retinal development is the SRY-related HMG-box (Sox) – Sox2 that promotes multipotency and self-renewal. Sox2 regulates the transcription of Notch1 and its conditional deletion results in a complete inhibition of neurogenesis where neither proneural genes nor neural differentiation markers are expressed (Taranova et al., 2006). The Müller glia express the transcription factors Sox2 and Pax6 and act as a true “stem cell” in the retina as they can produce rod precursors (Bernardos et al., 2007). The retinal progenitors also express progenitor proteins such as LIM domain homeobox proteins (Gong et al., 1995; Tétreault et al., 2009) that regulate the acquisition of cell fate.

Proneural genes

Proneural genes that encode bHLH transcription factors such as Atonal, achaete-scute complex like Ascl1 and Neurogenin Ngn1 (Korzh et al., 1998) initiate neurogenesis by regulating neuronal commitment, cell cycle exit, differentiation of retinal progenitors and activation of Notch signalling in neighbouring progenitors (Guillemot, 2007). Atonal has been shown to be required for photoreceptor R8 specification during drosophila eye development (Jarman et al., 1994; White and

Jarman, 2000). The wave of neuronal differentiation in the zebrafish retina is preceded by a wave of atoh7 expression, the zebrafish homologue of the Drosophila atonal (Jarman, 2000; Masai et al., 2000). Atoh7 is necessary for ganglion cell specification in the zebrafish and in neurogenesis in other vertebrates (Kanekar et al., 1997; Brown et al., 1998; McCabe et al., 1999; Kay et al., 2001). Furthermore, Pax6 has been shown to directly activate proneural genes Ascl1 and Neurog2 in the murine retina (Marquardt et al., 2001). Ascl1 is necessary in providing progenitor cells with competence for neuronal cells (Jasoni et al., 1994; Nelson et al., 2009).

Neuronal differentiation genes

Proneural proteins induce the expression of neuronal differentiation bHLH proteins such as NeuroD in postmitotic neurons that contributes to the neuronal differentiation programme. NeuroD is a homolog of Atonal and is specifically involved in cell fate determination of photoreceptors and amacrine cells (Yan and Wang, 1998; Morrow et al., 1999). Deletion of NeuroD in mice leads to impaired cone photoreceptor differentiation and degeneration of the rod photoreceptors (Liu et al., 2008). In zebrafish, rod precursors at a later stage in development and with the limited capacity to produce only rod photoreceptors express NeuroD (Hitchcock and Kakuk-Atkins, 2004). It has been implicated that combinations of bHLH and HD proteins in a context dependent manner act together to regulate specification of neuronal subtypes in the mouse and xenopus retina (Akagi et al., 2004; Hatakeyama and Kageyama, 2004; Ohsawa and Kageyama, 2008; Powell and Jarman, 2008). For instance, a combination of Mash1 and Chox10 expression is necessary for specifying a bipolar cell fate (Wang and Harris, 2005).

4.1.6 Extrinsic factors

Extrinsic signals can influence the fate of retinal progenitors at two different points. Firstly, post-mitotic neurons secrete soluble factors that control the cell fate

choices of the retinal progenitors via feedback inhibition as seen in amacrine cells (Belliveau and Cepko, 1999). Secondly, many factors have been shown to act on post-mitotic neurons that influnence their fate. For example, factors belonging to the ciliary neurotrophic factor (CNTF)/leukaemia inhibitory factor (LIF) family act on postmitotic neurons and drive rod precursors to attain a bipolar phenotype (Ezzeddine et al., 1997). In addition, signalling pathways such as hedgehog (Hh), Notch, RA, Wnt and FGF that can act positively or negatively to influence retinal neurogenesis. For detailed descriptions of the Notch and RA pathways, see general introduction. In the context of this chapter I will briefly highlight some of the contributions made to our understanding of signalling factors in retinal development.

Hedgehog signalling

Hedgehog signalling mediated through the secreted Hh protein is required for the promotion of photoreceptors in the Drosophila eye imaginal disc (Dominguez, 1999). Shh is expressed in different retinal neurons and is required for their differentiation in zebrafish retinal development. Similar to Drosophila development, the fan shaped differentiation wave observed during zebrafish neurogenesis is accompanied by a wave of sonic hedgehog (shh) expressed by the newly differentiating ganglion cells (Neumann and Nuesslein-Volhard, 2000; Shkumatava et al., 2004). Amacrine cells also express shh (Shkumatava et al., 2004) and the Hh signal from these sources is necessary for photoreceptor development.

Delta-Notch signalling

During development of the nervous system, Notch signalling promotes progenitor character at the expense of neuronal differentiation. Similarly, in zebrafish embryos, constitutively activated Notch1a expressed globally inhibits differentiation of retinal neurons but promotes the differentiation of Müller glia (Scheer et al., 2001). In the mind bomb (mib) mutant, Notch signalling is reduced

and causes a disruption in the differentiation of Müller glia and laminar organization (Bernardos et al., 2005).

Retinoic acid (RA)

Gain-of-function and loss-of-function studies have uncovered the role of retinoic acid in zebrafish eye morphogenesis. Exogenous application of RA induces duplication of the retina (Hyatt et al., 1992) while pharmacological reduction of RA synthesis can eliminate the formation of the ventral retina (Marsh-Armstrong et al., 1994). Interestingly, RA differentially influences opsin transcription in photoreceptors, regulates the differentiation and maturation but does not affect the fate of photoreceptors (Hyatt et al., 1996; Prabhudesai et al., 2005). This is in contrast to role of RA in determining photoreceptor fate in non-teleost vertebrates (Soderpalm et al., 2000).

Wnt signalling

Inhibition of the Wnt signalling pathway results in progenitor cells unable to incorporate bromodeoxyuridine (BrdU), thus revealing a role in promoting passage through the cell cycle (Masai et al., 2000). Interestingly, the Wnt pathway is antagonised by HDAC1 (*add*) to promote cell-cycle exit and neuronal differentiation (Yamaguchi et al., 2005).

FGF signalling

FGF plays a role early in development during patterning of the nasal-temporal retina (Picker and Brand, 2005) and in initiating the genesis of retinal neurons later in development (Martinez-Morales et al., 2005). The FGFs interact with tyrosine kinase receptors and activate the ras-MAPK pathway (Thisse and Thisse, 2005). Zebrafish have 25 defined FGF genes (Katoh and Katoh, 2005).

4.1.7 Continuous neurogenesis in the teleost retina

Retinal growth in adult fish has been studied for more than 50 years. The first study carried out was studying the development of the retina in the guppy *Lebistes reticulates* (Muller, 1952). Zebrafish similar to other teleost fish and amphibians grow throughout life. This is in contrast to the mammalian retina that stops growing after cessation of development. As the body grows, the eye size and the retinal area also increase (Hitchcock and Raymond, 2004). There are two mechanisms by which the retinal area is enlarged. Firstly, progenitor cells in the circumferential germinal zone (CGZ), a specialized area at the interface between the neural retina and the iris epithelium generate all types of retinal neurons. The newborn neurons at the periphery of the retina are integrated into the functional retina in an annular pattern (Otteson et al., 2002; Fadool, 2003). In the avian retina this process of ongoing neurogenesis also takes place to a limited extent (Fischer and Reh, 2000).

Secondly, the retina gradually stretches within the expanding optic cup, which reduces the packing density of the retinal neurons. As the increase in eye size helps to maintain visual acuity in the growing zebrafish, light sensitivity is preserved by the addition of new rod photoreceptors (Fernald, 1990). Rod photoreceptors are continuously produced from a population of mitotically active unipotent progenitors termed rod precursors. The rod progenitors were first described in goldfish and cichlids and have also been observed in other teleost species (Johns and Easter, 1977; Johns and Fernald, 1981; Raymond and Rivlin, 1987; Otteson and Hitchcock, 2003). These germinal progenitor cells are found interspersed in the ONL at the same level as the nuclei of the rod photoreceptors. The rod precursors originate from rounded and stationary slowly dividing cells in the INL, which give rise to intermediate fusiform-shaped cells that are radially arranged. The intermediate precursors migrate to the ONL along the radial processes of the Müller glia where they develop into the rod progenitors and subsequently into rod

photoreceptors (Johns, 1982) . This has also been confirmed in the rainbow trout and goldfish (Julian et al., 1998; Otteson et al., 2001).

The slow dividing cells in the INL express Pax6, whereas the rod progenitors are negative for the same but express NeuroD (Hitchcock and Kakuk-Atkins, 2004). Thus, suggesting that the proliferative cells in the INL are the "true" stem cells in the intact retina while the rod progenitors are classified as transit amplifying cells (Hitchcock et al., 1996; Otteson et al., 2001). Induction of the growth hormone/IGF-I signalling pathway induces proliferation of rod progenitors (Mack and Fernald, 1995), consistent with the view that rod photoreceptors are continuously added as the animal grows.

4.1.8 Retinal regeneration in the teleost retina

In amphibians, following retinal injury new neurons are added from the non-neuronal cells of the retinal pigmented epithelial layer (RPE). The RPE cells undergo a process of "regulated reprogramming" or dedifferentiation where they lose their pigment, re-enter the cell cycle and express genes intrinsic to retinal progenitors during embryonic development (Okada, 1980; Bermingham-McDonogh and Reh, 2011). Zebrafish also have a significant capacity to regenerate retinal tissue following injury. Several studies of retinal regeneration have involved different methods to damage retinal neurons in teleost fish. Surgical removal of a piece of retina (Cameron, 2000; Cameron et al., 2005; Yurco and Cameron, 2005), cytotoxic damage using chemicals such as ouabain, 6-hydroxydopamine and tunicamycin (Braisted and Raymond, 1992, 1993), laser ablation (Braisted et al., 1994; Wu et al., 2001), photoxicity (Vihtelic and Hyde, 2000; Vihtelic et al., 2006) and heat lesioning (Raymond et al., 2006) induced proliferation and regeneration in the damaged retina.

Müller glial cells that are quiescent in the adult retina are activated following injury during which they proliferate and produce INL stem cells (Wu et al., 2001; Fausett

and Goldman, 2006). Müller glia dedifferentiate in a process similar to that described in amphibians and re-express many genes expressed by embryonic retinal progenitors (Fausett and Goldman, 2006; Raymond et al., 2006; Bernardos et al., 2007; Thummel et al., 2008; Qin et al., 2009; Ramachandran et al., 2010). Rod precursors also contribute to retinal regeneration, especially when rod precursors are damaged.

In mammals, there is little or no regeneration of retinal tissue following injury. Müller glia in rodents become "reactive", similar to the astrocytic response observed following neuronal damage in other regions of the central nervous system. They increase their expression of GFAP and very few enter the cell cycle and proliferate (Dyer and Cepko, 2000; Karl and Reh, 2010). When treated with mitogenic proteins such as EGF, IGF, FGF, Wnt 3a after retinal damage some Müller glia proliferate. The barrier on Müller glial proliferation is the inhibitor Cdki, $p27^{kip1}$ that is activated by TGF-beta (Close et al., 2005; Close et al., 2006; Karl et al., 2008). Even under such conditions, the Müller glia does not express the proneural genes Ascl1 and Ngn2. Thus, mammalian progenitors undergo only a partial "reprogramming" in contrast to that seen in the zebrafish (Bermingham-McDonogh and Reh, 2011).

Since zebrafish have an incredible capacity to regenerate damaged retinal neurons they have been used in several studies aiming to identify the molecular mechanisms necessary for regeneration, with a view to identifying therapeutic targets in order to treat retinal diseases in humans. Several genes have been shown to be important for successful retinal regeneration. Knockdown of PCNA and the bHLH transcription factor Ascl1 inhibits retinal regeneration (Fausett et al., 2008; Thummel et al., 2008). In addition, it has been shown that CNTF is expressed during regeneration and is necessary for Müller glia proliferation (Kassen et al., 2009). In post-hatch chicks retinal damage results in a tempered response of Müller glia proliferation, re-expression of some developmental proneural genes and a

limited amount of neuronal regeneration (Fischer and Reh, 2001; Fischer et al., 2002; Fischer and Reh, 2003).

Injury-induced re-expression of developmental proneural genes, plus Delta-Notch signalling could contribute to retinal progenitor proliferation and regeneration in the zebrafish retina (Yurco and Cameron, 2007). Consistent with this view, it has been found in the avian retina that blocking Notch signalling prior to regeneration inhibits Müller glia from re-entry into the cell cycle. In contrast, inhibiting Notch signalling after injury-induced proliferation, promotes differentiation into amacrine cells (Hayes et al., 2007; Ghai et al., 2010). In summary, zebrafish exhibit some highly conserved developmental processes to those seen in other vertebrates during retinal development.

4.1.9 Experimental objectives and hypotheses

In this chapter, I report an interesting finding where overactivation of Notch signalling in the uninjured adult retina leads to a large eye phenotype in transgenic heatshocked retinae. To establish the causality of the size increase I immunostained retinal sections from transgenic heatshocked fish with PCNA, a marker for cells in the G1 and S phases of the cell cycle. For comparison, I also immunostained sections from transgenic non-heatshocked and wildtype heatshocked retinae to check if the presence of the transgene or heatshocks per se contributed to the increase in eye size. I counted the number of PCNA positive nuclei in the INL and ONL in the retina. In addition, I also immunolabelled the active Notch that is myc tagged to determine if the heatshock did activate Notch and its pattern of expression. **I hypothesized that the heatshock would induce Notch expression in all cells in the retina and the activation would increase proliferation in the uninjured retina causing it to grow.**

Next, I aimed to determine if the increase in proliferation leads to an increase in retinal length. Cross-sections of transgenic heatshocked retinae showed many

indentations in comparison to transgenic non-heatshocked and wildtype heatshocked retinae. I measured the total retinal length in these cross-sections in all three groups. **I hypothesized that in increase in proliferation would increase the total retinal length due to addition of new cells.** I then set out to investigate if Notch overexpression has any effect in the uninjured adult brain. Similar to the retinal immunolabellings, I stained brain cross-sections of transgenic heatshocked, transgenic non-heatshocked and wildtype heatshocked retinae for active Notch expression and proliferation. Given that Notch has been found to be a negative regulator of neurogenesis while maintaining the progenitor pool in the adult brain (Chapouton et al., 2010). **I hypothesized that following overexpression of Notch progenitor cells would be pushed back into quiescience and hence there would not be an increase in proliferation.**

Finally, I asked the question whether inhibition of Notch signalling with a γ-secretase inhibitor DAPT would influence proliferation in the uninjured adult retina. I treated wildtype fish with DAPT, injected intraperitoneally and immunolabelled retinal sections with PCNA. As per my initial hypothesis that Notch promotes proliferation in the retina then it would be tempting to speculate that inhibition of Notch activity would result in the opposite outcome and drive progenitors back into quiescience. In the normal adult zebrafish retina, proliferation in the central retina is sparse and new neurons are continuously added to the intact retina from the circumferential germinal zone at the periphery (Otteson et al., 2002; Fadool, 2003). **Taken together, I hypothesized that there would no detectable effect on proliferation after inhibition of Notch activity.**

4.2 Results

4.2.1 Notch over-activation strongly induces an increase in eye size and proliferation in the inner nuclear layer of the retina

Notch signalling can be spatially and temporally over-activated in double-transgenic fish, *Tg(hsp70l:Gal4) x Tg(UAS:myc-notch1a-intra)*, where the heat-shock promoter can drive the expression of the active intracellular domain of *notch1a,* fused to a myc epitope (Scheer et al., 2001). Over-activation of Notch in the adult lesioned spinal cord was found to strongly inhibit the generation of motor neurons and attenuated proliferation of progenitor cells as described in the previous chapter. Incidentally observed and in contrast, the same manipulation induced a robust increase in eye size after heat-shocks in uninjured transgenic and not in heat-shocked wildtype or non-heat-shocked transgenic animals (Fig. 4.2).

To determine the reason behind the increase in eye size after heat-shocks in uninjured transgenic animals, I used proliferating cell nuclear antigen (PCNA) immunohistochemistry, which labels cells in the G1 and S phase of the cell cycle. My hypothesis was that an increase in proliferation in the retina could at least in part, contribute to the increase in eye size. In all experiments, I analysed the region around the optic nerve head, where the optic nerve exits the eye, which provides a size-independent landmark. I quantified the number of PCNA immunoreactive profiles in the outer and inner nuclear layer in a 920μm area from the CGZ. I found a strong increase in the number of PCNA positive nuclei in the inner nuclear layer but not in the outer nuclear layer in retinae of transgenic heat-shocked fish (Fig. 4.3). A striking difference between transgenic heat-shocked retinae and the controls was the complete detachment of the RPE. This phenomenon was observed when retinae were microdissected out of the eyeball for tissue sectioning. It is also

noteworthy to mention that wildtype heat-shocked retinae displayed an increase in PCNA positive nuclei in the ONL suggesting that the heat-shock alone has a strong effect on proliferation even in the absence of activated Notch.

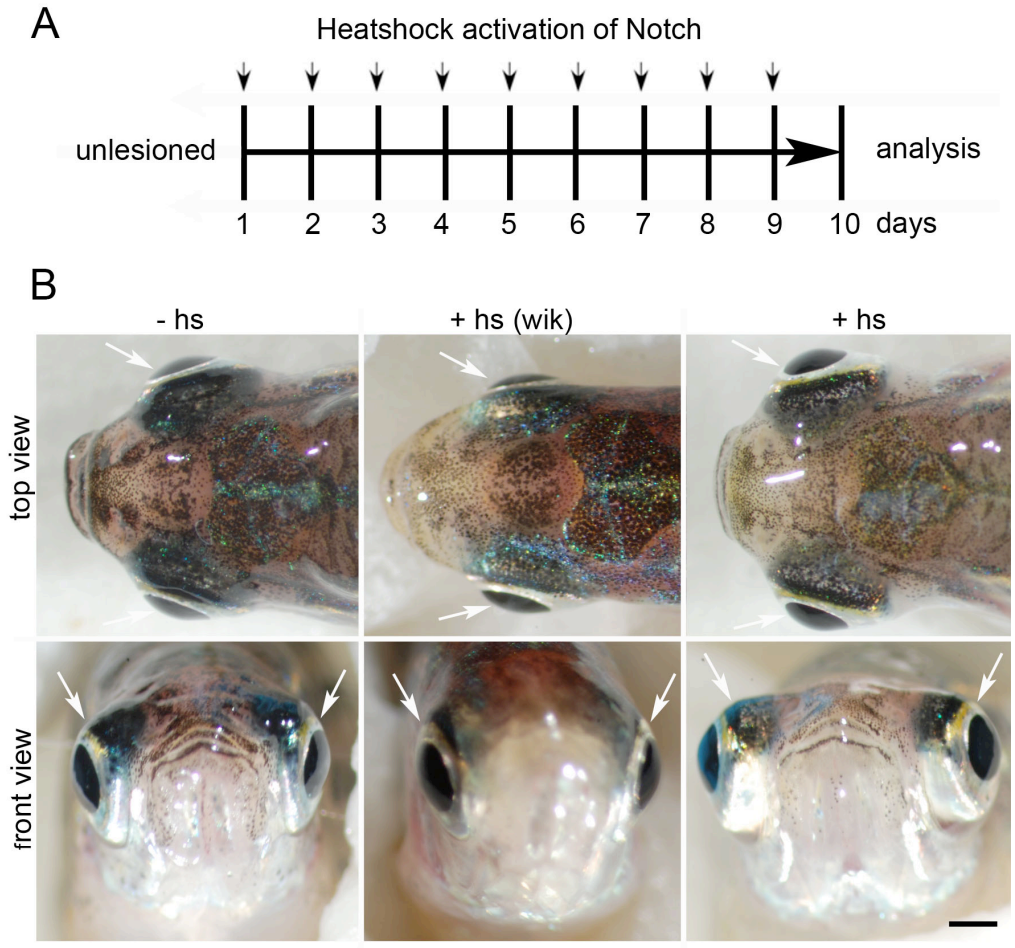

Fig 4.2 Over-activation of Notch activity induces an increase in eye size. **A**. Schematic of the timeline used for administration of heat-shocks in uninjured animals. **B**. Top and front stereomicroscopic views of eyes *in situ* shows a strong increase in size in heat-shocked animals compared to non-heat-shocked transgenic and heat-shocked wild-type/wik controls (n=3). Arrows denote eyes in animals. – hs denotes non-heat-shocked transgenic and +hs denotes heat-shocked transgenic animals. Scale bar in B = 100µm

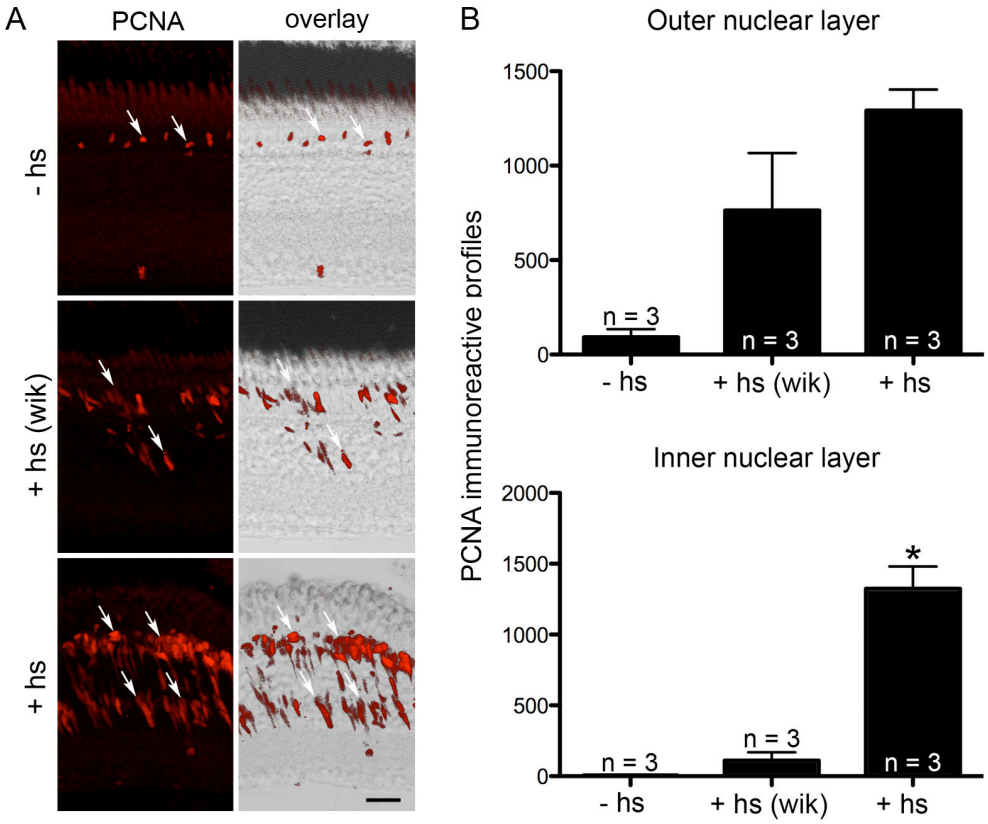

Fig 4.3 Forced activation of Notch signalling induces a strong increase in proliferation. **A**. PCNA immunohistochemistry in retinal cross-sections reveals a strong increase in the number of labelled nuclei in the transgenic heat-shocked animals compared to the transgenic non-heat-shocked animals and wild-type/wik heat-shocked animals (n =3). Arrows indicate PCNA immunoreactive nuclei. **B**. The number of PCNA positive nuclei in the inner nuclei layer (*$P < 0.01$) but not in the outer nuclear layer ($P > 0.05$) is strongly increased. – hs denotes non-heat-shocked transgenic and +hs denotes heat-shocked transgenic animals. Scale bar in A = 50µm

4.2.2 Retinal length is not increased after heat-shock administration

Preliminary observation of the retinal sections revealed that in heat-shocked animals, the retinae had more indentations than the non-heat-shocked transgenic and heat-shocked wild-type controls (n = 3; Fig. 4.4A). However, when the total length of the retina in a single optical section was measured, I found no significant increase after heat-shocks (transgenic non-heat-shocked animals: 2905 ± 157.5, n =

3; wild-type heatshocked animals: 2892 ± 54.5, n = 3; transgenic heat-shocked animals: 3452 ± 173.9, n = 3; P > 0.05; Fig. 4.4B). It is highly unlikely that an increase in proliferation would not correspond to a subsequent increase in eye size. We would need to increase the number of animals analysed to confirm if retinal length increases after Notch over-activation using this method of measurement. Furthermore, we would like to quantify the retinal area in flat-mounts of the retina.

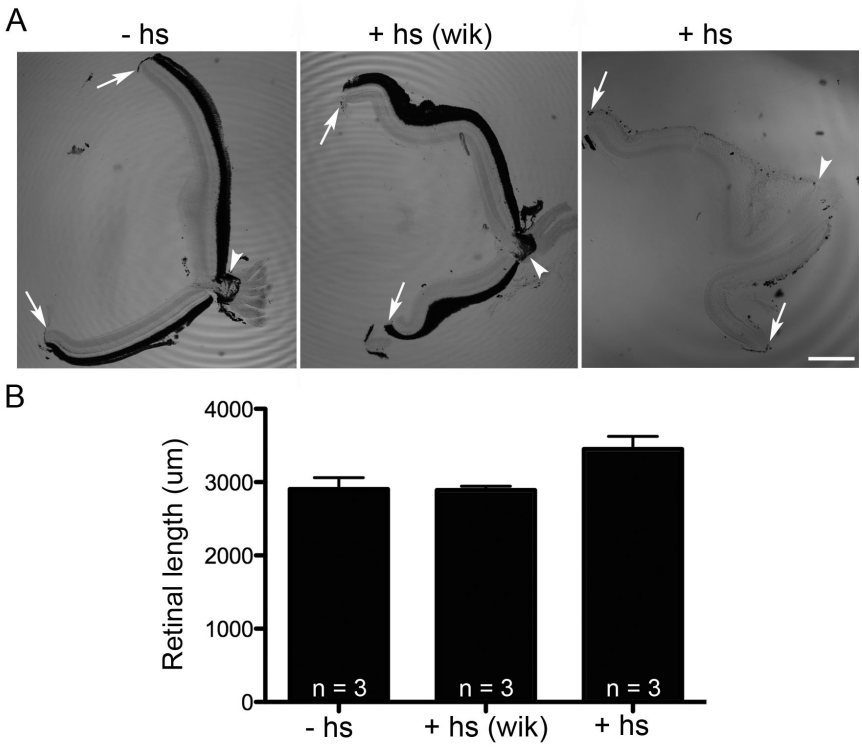

Fig 4.4 Length of retina after heat-shocks is not increased. A. Images of single optical coronal sections of the retina with the optic nerve attached were used to measure the total retinal length. The length was measured from one end of the retina to the other. Arrows denote the tips at the ends of the retina from where the length was measured. Arrowheads mark the optic nerve. B. The length of the retina (measured in µm) following heat-shocks is not significantly increased compared to transgenic non-heat-shocked and heat-shocked wild-type/wik control animals. – hs denotes non-heat-shocked transgenic and +hs denotes heat-shocked transgenic animals. Scale bar = 200µm.

4.2.3 The intracellular domain of Notch is over-expressed in all cell layers in the heat-shocked retina

Upon heatshock administration in the unlesioned and lesioned spinal cord, the myc tagged intracellular domain of Notch is expressed in the nuclei of ventricular ependymal cells and in their radial processes. However, in the retina, the active Notch as detected by myc immunohistochemistry was widely expressed in all cellular layers in the transgenic heat-shocked animals in comparison to no detectable expression in the non-heat-shocked and heat-shocked wild-type control. Myc immunoreactivity was also observed in the processes and end feet of presumptive Müller glial cells in the inner and outer limiting membranes respectively (n = 3; Fig 4.5). Thus, the heatshock alone can activate Notch signalling in the uninjured retina.

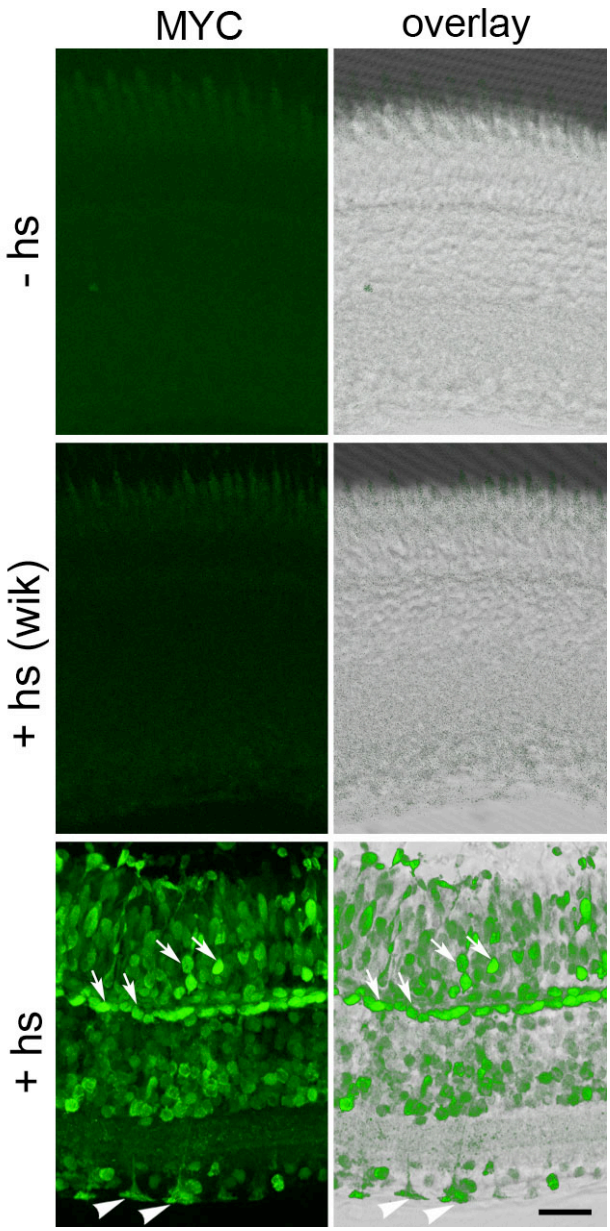

Fig 4.5. Active component of Notch is widely over-expressed in the uninjured retina. Myc immunohistochemistry in cross-sections of the retina shows myc expression in the heat-shocked retina as opposed to no expression in the controls. Arrows denote cells in the outer and inner nuclear layers expressing myc. Arrowheads mark the end feet of presumptive Müller glial cells. – hs denotes non-heat-shocked transgenic, +hs denotes heat-shocked transgenic animals and wik denotes heatshocked wild-type animals. Scale bar = 50µm

4.2.4 Notch activation has no effect on proliferation in the uninjured brain

Similar to the mammalian brain, the zebrafish brain also contains active neurogenic niches where continuous neurogenesis takes place (Chapouton et al., 2007; Zupanc, 2008; Kaslin et al., 2009). Given that forced activation of Notch activity in the retina induces a robust increase in eye size and proliferation, the same mechanism could act on the brain, increasing its size. To test if this is the case, we used PCNA immunohistochemistry in coronal sections of the forebrain and the hindbrain (data not shown). Qualitatively, there was no observable difference in the amount of PCNA positive nuclei in the transgenic heat-shocked animals and the controls (n = 3; Fig. 4.6A). Myc immunoreactivity was strong in forebrain ependymal progenitor cells and their radial processes. We observed immunoreactivity for myc in non-heat-shocked transgenic animals and in heat-shocked wild-type fish. Since, wild-type fish do not express the Notch-intra-myc transgene, we believe that our observation is due to non-specific cross-reactivity of the anti-myc antibody (n = 3; Fig. 4.6B).

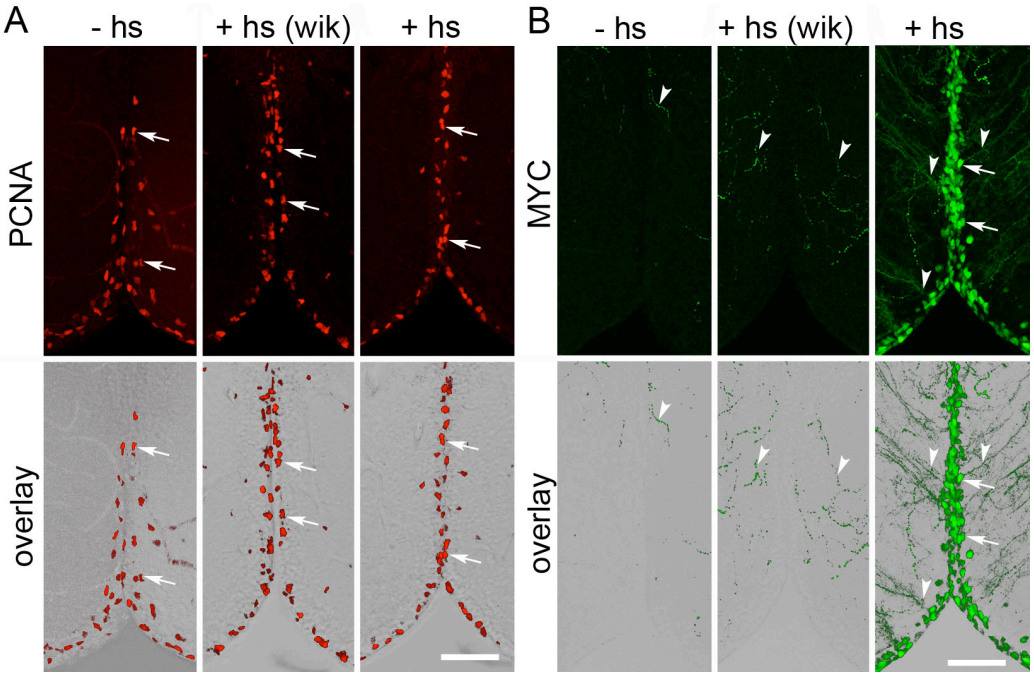

Fig 4.6. Notch activation in the uninjured brain has no apparent effect on proliferation. Cross-sections of the forebrain are shown (dorsal is up). **A.** Activation of the Notch pathway has no apparent effect on proliferation as detected by PCNA immunohistochemistry in the transgenic heat-shocked animals compared to the transgenic non-heat-shocked and wild-type/wik heat-shocked control brains. Arrows indicate PCNA immunoreactive cells. **B**. Notch1a-inta-myc, the active component of the Notch pathway, is expressed in forebrain ependymal progenitor cells and their radial processes in transgenic animals after heat-shocks. Myc immunoreactivity in processes of non-heat-shocked and heat-shocked wild-type animals is likely due to non-specific cross-reactivity. Arrows denote forebrain ependymal cells that express the activated protein and arrowheads mark myc expression in radial processes. – hs denotes non-heat-shocked transgenic and +hs denotes heat-shocked transgenic animals. Scale bar in A = 50µm and in B = 50µm

4.2.5 DAPT has no effect on proliferation in the uninjured retina

DAPT is a widely used small molecule that inhibits the γ-secretase complex from cleaving the Notch receptor and thus blocks Notch signalling. Injected intraperitoneally, DAPT significantly enhanced the generation of motor neurons and subsequent proliferation in the lesioned spinal cord. In the unlesioned spinal cord where there is little or no neurogenesis (Reimer et al., 2008), DAPT had no effect on proliferation or on motor neuron generation. Similarly, with the same

manipulation (Fig. 4.7A), DAPT had no effect on the proliferation of retinal cells in the outer or inner nuclear layers compared to DMSO treated animals (Fig. 4.7B,C). Thus, inhibiting Notch signalling does not promote cycle exit of retinal progenitors.

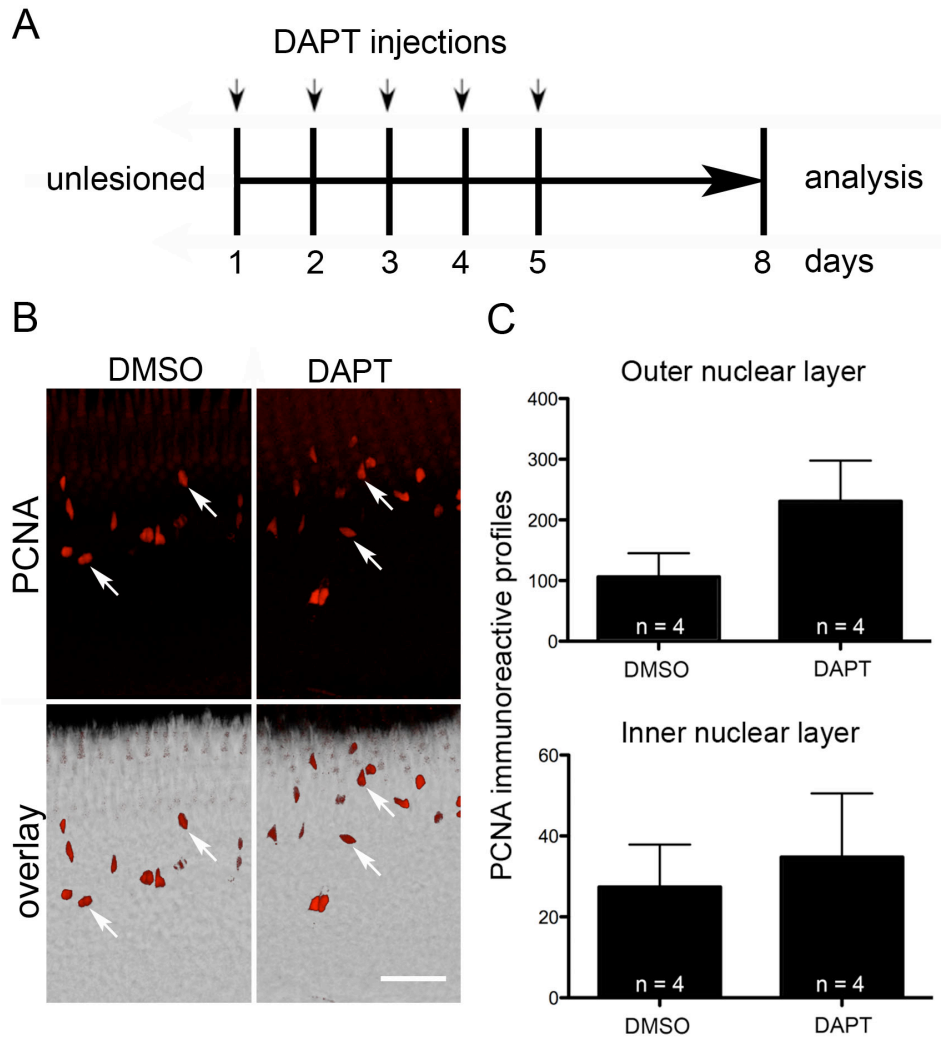

Fig 4.7. Inhibiting Notch activity with DAPT has no effect on proliferation in the uninjured retina. **A**. Schematic of the timeline used for DAPT injections. **B**. PCNA immunohistochemistry in cross-sections of the retina reveals proliferative cells in the outer and inner nuclear layers. Arrows mark PCNA positive nuclei. C. The number of PCNA immunoreactive cells in the nuclear layers were unaffected by DAPT injections compared to DMSO treated animals ($P > 0.05$). Scale bar in B = 25µm

4.3 Discussion

In this chapter, I have presented preliminary observations of an incidental find involving the effects of Notch signalling in the uninjured adult zebrafish retina. The function of Notch signalling in retinal development has been extensively studied, however few studies have focussed on its role in the intact adult retina. Unlike the mammalian retina that stops growing after development is complete, neurogenesis of retinal neurons persists throughout the life of a zebrafish. As the body of the animal grows, its eye size and retinal area also increases (Hitchcock and Raymond, 2004). Since the retina grows in concentric annuli, whereby more proliferative activity is observed near the retinal margins in the "younger" part of the retina than in the "older" central part, proliferation in the central retina is sparse (Johns, 1982).

4.3.1 Notch activation leads to an increase in eye size and proliferation

I find that forced expression of active Notch using the strong Gal4/UAS activator system (Scheer and Campos-Ortega, 1999; Takke et al., 1999) in the intact adult retina, leads to a strong increase in eye size and proliferation in the INL in the central retina. This effect may be attributed to Notch activity alone and not the heat-shocks as eye size is not increased in transgenic non-heatshocked and wild-type heatshocked controls. However, we cannot ignore the strong effect exerted by the heat-shock alone on proliferation in the ONL as seen in wild-type heatshocked controls. Damage or loss of the RPE results in rod photoreceptor cell death and loss of vision (Sparrow et al., 2010). It is therefore plausible that the heatshock serves as a thermal lesion and damages the RPE resulting in loss of rod photoreceptors. Rods are continuously replaced by actively proliferating unipotent rod progenitors, which are found interspersed in clusters in the ONL. Immunolabelling with PCNA in wild-type heatshocked retinae reveal putative proliferating rod progenitors in the ONL that respond to the damage. Interestingly, a chronic amount of rod cell death

stimulates regeneration from the rod progenitors rather than the Müller glia in the INL (Montgomery et al., 2010).

An alternative hypothesis for the 'large eye' phenotype of transgenic heat-shocked zebrafish, could be the knock-on toxic effects of the heatshock on the RPE and activated Notch promoting proliferation in the INL. Together the heatshock and Notch overexpression might lead to the massive increase in proliferation in the central retina and eye size. Importantly, we cannot exclude any toxic effects of the transgene itself and it is possible that the toxic effects of the heatshock and/or activated Notch contribute to the damage in the RPE and/or rod photoreceptors. If this were the case then the increase in proliferation would be secondary consequences of such toxicity and would reflect regeneration triggered by the loss of the RPE and rods.

Nevertheless, the normal layered organisation of the retina after heat-shocks was clearly distinguishable although, we would still need to stain retinal sections with neutral red to confirm this observation. Activated Notch as detected by myc immunohistochemistry was widely expressed in all nuclear layers of the retina following heat-shocks. The heat-shock alone could trigger the expression of the active Notch in the retina. It would be informative to examine the expression of Her4.1, a downstream target gene of Notch1a in heat-shocked retinae. We have previously shown that over-expression of Notch in the unlesioned and lesioned spinal cord of adult zebrafish leads to an increase in Her4.1 expression.

4.3.2 Notch may influence neurogenesis in the uninjured adult retina

Müller glia occupy the INL and are slowly dividing retinal "stem cells" that actively proliferate in response to injury (Raymond et al., 2006; Bernardos et al., 2007). Given that expression of activated Notch promotes proliferation in the INL and that the morphological features of PCNA immunoreactive cells in the INL

resemble Müller glia, we propose that Notch influences proliferation of presumable Müller glia in the undamaged adult retina.

Since Müller glia are multipotent progenitors and can generate all retinal neurons, it is tempting to speculate that other cell types in the INL such as bipolar, amacrine, horizontal and ganglion cells in the GCL are being new born (Bernardos et al., 2007). As Müller glia express glutamine synthetase and glial fibrillary acidic protein (GFAP) (Peterson et al., 2001), I will perform immunohistochemistry to detect these antigens in order to confirm our hypothesis that the proliferating cells are indeed Müller glia. Amacrine cells express serotonin (5-HT) and dopamine (TH) whereas ganglion cells express zn-8 (neurolin). Subsequently, we will use immunohistochemistry and the Click-iT® EdU kit (Invitrogen, UK) to identify if amacrine cells and retinal ganglion neurons are newly generated.

It would also be informative to investigate if the increased proliferation following Notch activation is associated with any amount of cell death. I will use 4C4, a marker for macrophages/microglia to identify interactions with proliferating cells and/or newly generated retinal neurons.

4.3.3 Notch activation and the cell cycle

Notch signalling together with cyclin-dependent kinase inhibitors has been shown to regulate cell cycle progression and promotion of glial cell fates. Ohnuma et al., 1999 showed that mis-expression of $p27^{Xic1}$ of the Cip/Kip family of Cdk (cyclin-dependent kinases) inhibitors in *Xenopus* together with expression of activated Notch resulted in an increase in the number of Müller glia. They speculated that $p27^{Xic1}$ and Delta-Notch signalling together promote Müller glial cell fate. In addition, Notch-regulated cyclin-dependent kinase inhibitor function is necessary for oligodendrocyte specification in the zebrafish spinal cord (Park et al., 2005). Since Notch activation induces proliferation in the intact adult retina it would be interesting to examine the expression of the cyclin-dependent kinase inhibitors.

4.3.4 Notch activation during retinal development

In the developing zebrafish retina, Notch activation led to a decrease in eye size and a strong reduction in the number of proliferative cells in the central region of the retina. Consequently, striking pattern defects were observed where the characteristic palisade-like arrangement of the photoreceptor outer segments and the plexiform layers could not be distinguished (Scheer et al., 2001). Interestingly, forced activation of Notch, Hes1 or Dl1 in the embryonic retina of mice and chick show that neuronal differentiation is inhibited while proliferating progenitors are maintained (Bao and Cepko, 1997; Henrique et al., 1997; Furukawa et al., 2000; Jadhav et al., 2006).

Furthermore, a recent study showed that forced activation of Notch in retinal pigmented epithelial (RPE) cells in the developing rodent retina resulted in a strong reduction in eye size or microphthalmia. The activated Notch maintained RPE cells in a hyperproliferative and undifferentiated state that resulted in benign, pigmented tumours. The neural retina was severely damaged and in some animals it was absent altogether (Schouwey et al., 2010).

Taken together, these results suggest that Notch inhibits neuronal differentiation during embryonic retinal development and leads to a reduction in eye size associated with aberrant morphologies. This suggests a role for Notch in the maintenance of progenitor character. However, in some cases Notch can promote hyperproliferation leading to the formation of tumours.

4.3.5 Role of Notch in adult progenitor pools

A spinal cord lesion induces proliferation of spinal cord progenitors and the generation of motor neurons (Reimer et al., 2008). We have demonstrated in Chapter 1 that in the adult lesioned spinal cord, forced activation of Notch attenuates proliferation of spinal cord progenitors and the generation of motor

neurons from these cells. In contrast, there is little or no proliferation in the unlesioned spinal cord (Reimer et al., 2008) and expression of activated Notch had no effect on motor neuron generation or proliferation.

In the adult zebrafish forebrain, constitutive proliferation of progenitor cells and subsequent neurogenesis occurs in specific germinal zones (Chapouton et al., 2007; Kaslin et al., 2008). Proliferation in these neurogenic zones was unaffected by Notch activation. This is consistent to published observations (Chapouton et al., 2010). In the adult zebrafish retina there is ongoing production of retinal neurons from the CGZ at the retinal margins and the continuous replacement of rod photoreceptors in the ONL. However, proliferation in the central retina is sparse but on over-expression of Notch the overall eye size and proliferation in this region increases significantly.

In summary, the anti-neurogenic activity of Notch in the lesioned spinal cord is consistent to its role during constitutive neurogenesis in the zebrafish forebrain (Chapouton et al., 2010). However, this is contrast to the adult unlesioned zebrafish spinal cord. Strikingly, in the uninjured adult zebrafish retina, Notch promotes proliferation of presumptive Müller glia and possible neuronal differentiation contrary to its traditional inhibitory role. Activated Notch signalling promotes proliferation in the *Drosophila* midgut (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) and the mammalian epidermis (Lowell et al., 2000; Nicolas et al., 2003) similar to our observations in the uninjured adult zebrafish retina. Interestingly, stem cells in the interfollicular regions of the epidermis and the insect midgut express the Notch ligands which activates Notch signalling in the neighbouring cells. So, instead of maintaining a progenitor-like state, Notch drives the cells towards a post-mitotic fate and terminal differentiation.

In the injured retina, cells in the CMZ and INL are immunopositive for the Notch ligand deltaC and colocalize with BrdU, a marker for proliferation. Cells expressing Notch receptors did not co-localize with deltaC immunoreactivity or

BrdU label and were found in adjacent cells (Raymond et al., 2006). This strongly suggests that the Notch ligand is expressed in proliferating retinal progenitors which activates Notch signalling in the neighbouring cells. It would be informative to determine if retinal stem cells express Notch ligands in the uninjured and transgenic heatshocked retinae. If yes, then activation of Notch should drive the cells into a post-mitotic fate. Thus in this context, Notch would serve as a positive regulator of neurogenesis in the zebrafish retina.

Inhibition of Notch signalling using the γ-secretase inhibitor DAPT in the uninjured adult retina did not have any effect on proliferation or eye size. There are several possible reasons to explain this result. Firstly, it is possible that the DAPT inhibition in the retina did not work probably due to the amount of DAPT that reached the retina after being administered intraperitoneally. However, in future experiments this should be confirmed by analyzing the downregulation of the target gene her4.1. A study done in adult newts detailing the role of Notch signalling in the retina, successfully administered the DAPT by intraocular injections and observed a subsequent downregulation of Hes1 (Nakamura and Chiba, 2007). Secondly, following our hypothesis that Notch activation promotes proliferation in the adult retina then one would expect that inhibiting Notch would give the opposite result. Speculatively, Notch inhibition could drive retinal progenitors back into quiescience. Finally, it is also possible that loss of Notch activity could promote uncontrolled proliferation instead of pushing the stem cells back into quiescience as reported in the *Drosophila* midgut (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006) and mammalian epidermis (Lowell et al., 2000; Nicolas et al., 2003). Extrapolating from the Notch activation experiments, Notch could act as a positive regulator of neurogenesis in the normal zebrafish retina but further experiments need to be done to confirm this view.

A recent study showed that adult human Müller glia retain an unpredicted plasticity and multipotent potential capable of generating rod photoreceptors, thus making

Müller glia a promising source of novel therapeutic applications in treating retinal injury (Giannelli et al., 2011). However, further investigations of Notch-induced neurogenesis in the adult zebrafish retina could potentiate endogenous retinal repair in humans.

5. Additional signaling pathways are involved in motor neuron regeneration

5.1 Introduction

In the adult mammalian spinal cord, ependymal cells with radial processes lining the central canal are recruited to proliferate in response to a lesion. However, these cells ultimately adopt a glial fate and contribute to a glial-scar (Meletis et al., 2008). Interestingly, these adult stem cells, when transplanted into the dentate gyrus, generate neurons (Shihabuddin et al., 2000). Consequently, it is pivotal to elucidate the molecular mechanisms in spinal cord stem cells, the environmental signals in the adult niche and signalling pathways that regulate adult neurogenesis. The insights gained would help to better inform therapeutic targets or strategies in replacing lost or damaged motor neurons in spinal cord injury or neurodegenerative diseases such as amyotrophic lateral sclerosis (Lindvall and Kokaia, 2010).

The adult brain in mammals and zebrafish has specified constitutively active neurogenic zones that continuously generate neurons (Chapouton et al., 2007; Gould, 2007; Kaslin et al., 2009; Chapouton et al., 2011). In contrast, the uninjured mammalian spinal cord has little no constitutive neurogenesis similar to the zebrafish spinal cord (Meletis et al., 2008; Reimer et al., 2008). Remarkably, zebrafish can replace lost motor neurons from quiescent progenitors following a complete transection of the spinal cord and are capable of successful spinal cord regeneration (Becker et al., 2004; Reimer et al., 2008). The spinal progenitors are morphologically similar to those seen in the adult mammalian spinal cord (Meletis et al., 2008). Furthermore, they retain developmental characteristics of motor neuron progenitors and are competent to respond to the floor plate derived morphogen shh in generating new motor neurons (Fuccillo et al., 2006; Reimer et al., 2009).

5.1.1 Retinoic acid signalling during spinal cord regeneration

Genes integral to RA signalling, namely the RA receptor subunits (rarab, rxrγa,

rxrγb) and downstream genes (crabp2a, cyp26a), are robustly upregulated as assessed by PCR following a spinal cord lesion at two weeks post lesion. Thus, implying that RA signalling has a putative role in motor neuron regeneration (Reimer et al., 2009). I used *in situ* hybridisation for candidate RA pathway genes to determine their expression patterns in the adult lesioned spinal cord. I found that the RA catabolising enzyme, cyp26a was upregulated predominantly in ventricular spinal progenitor cells in the lesioned spinal cord. In contrast, crabp2a, a cellular retinoic acid binding protein was upregulated rostral to the lesion site in one or two cells in the ventro-lateral domain of the lesioned spinal cord.

5.1.2 Dopamine signalling in motor neuron development and regeneration

Local signals such as shh and RA are known to influence the generation of various cell types during the development of the ventral spinal cord (Fuccillo et al., 2006; Maden, 2006). However, signals from long-range projection axons also influence cell proliferation in target regions. In the cortex, thalamocortical axons increase mitotic activity (Dehay et al., 2001) and olfactory axons influence cell cycle length in the olfactory bulb primordium (Gong and Shipley, 1995). Therefore, it is likely that signals from descending axons may influence motor neurogenesis in the ventral spinal cord.

Dopaminergic projection axons exclusively derived from diencephalic dopaminergic neurons innervate the embryonic zebrafish spinal cord during motor neuron differentiation serving as the only source of dopamine and as an excellent candidate for such a signal (McLean and Fetcho, 2004b, a). Dopamine signalling has been implicated in influencing developmental (Ohtani et al., 2003; Popolo et al., 2004) and adult neurogenesis (Borta and Höglinger, 2007). However, this role of dopamine is unclear and is highly debated. Some studies support the view that dopamine influences proliferation of neural stem cells and promotes adult

neurogenesis (Baker et al., 2004; Hoglinger et al., 2004) while others suggest that it has an inhibitory action (Kippin et al., 2005; Peng et al., 2008; Park and Enikolopov, 2010). It is important to elucidate how a long-range signal like dopamine would interact with local signalling pathways in the ventral spinal cord to control motor neuron differentiation.

Here we show using pharmacological and morpholino knockdown experiments that brain-derived dopamine augments the generation of motor neurons during spinal cord development and regeneration. Amongst the D2-like receptors, we identified the activation of the D4a receptor and a subsequent increase in hedgehog signalling. These studies show for the first time that descending axons influence the plasticity of adult spinal progenitors and regulate subsequent neurogenesis in the spinal cord. We envisage that this newly discovered signalling mechanism that can be activated by drugs might provide promising leads for promoting neurogenesis in motor neuron disease or after spinal cord injury.

Taken together, we show that the downstream genes of RA, cyp26a and crabp2a are differentially expressed in ventricular progenitor cells and a sub-population of spinal neurons i.e presumptive motor neurons respectively, thus suggesting a role for RA in motor neuron regeneration. We also show that brain-derived dopamine activates the D4a receptor and subsequent hedgehog signalling to influence the generation of motor neurons during embryonic development and in the adult lesioned spinal cord.

5.2 Results

5.2.1 Signalling components of the RA pathway are upregulated after a lesion

It has been previously shown by PCR only that various signalling components of the RA pathway are upregulated following a spinal cord lesion suggesting a role

for RA signalling in motor neuron regeneration (Reimer et al., 2009). The mRNA levels of several genes of the RA pathway namely retinoic acid receptor subunits (rarab, rxrγa, rxrγb) and downstream genes (crabp2a, cyp26a) were robustly upregulated (Fig. 5.1). However, the retinoic acid synthesizing enzyme raldh2, other receptor subunits (raraa, rarγa, rarγb, rxraa) and the downstream target gene crabp2b were not upregulated.

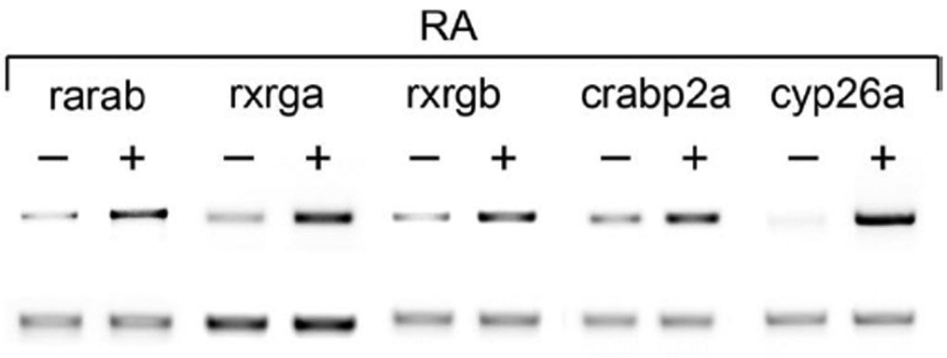

Fig. 5.1 RA genes are upregulated after a lesion. Genes of the RA pathway are upregulated in the lesioned spinal cord as assessed by PCR. - = unlesioned and + = lesioned. This figure was taken from (Reimer et al., 2009).

In order to determine the expression patterns of the RA genes found to be upregulated by PCR in the lesioned spinal cord, I cloned the RA receptor subunits (rarab, rxrγa, rxrγb) and downstream genes (crabp2a, cyp26a) into expression vectors to synthesize antisense probes and used *in situ* hybridisation for the candidate genes. The quality of the probes was initially validated on 24hpf wild-type zebrafish embryos. Cyp26a, was expressed in the notochord and tail bud whereas rarab was expressed in the CNS and the immature eye (n = 10). The retinoic acid receptor alpha b (rarab) was widely expressed in the central nervous system and the embryonic eye (n = 10). Retinoid X receptors gamma a (rxrga) and gamma b (rxrgb) were expressed in the anterior spinal cord (n = 10; Fig. 5.2). The in situ expression patterns for these genes were consistent and in accordance with published results. Thus confirming the accuracy and quality of the mRNA probes.

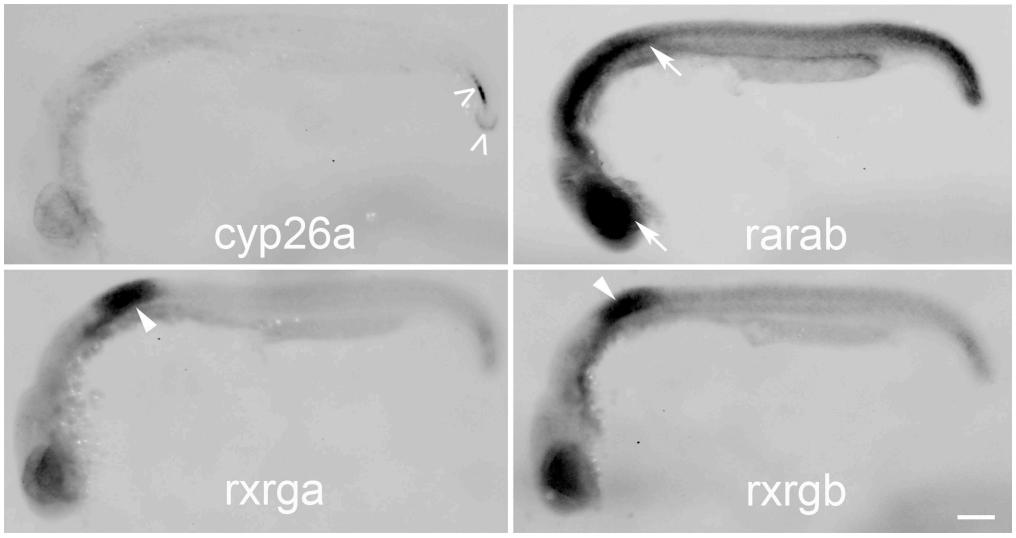

Fig. 5.2 mRNA probes for RA genes are validated using whole mount in situ hybridization. In situ hybridizations in 24hpf wild-type zebrafish embryos are shown here; open arrowheads indicate expression in the notochord and the tail bud, arrows point to expression in the CNS and eye whereas arrowheads denote expression in the anterior spinal cord. Retinoic acid catabolizing enzyme, Cyp26a was expressed in the notochord and tail bud. Rarab was widely expressed in the CNS and eye whereas rxrga and rxrgb were strongly expressed in the anterior spinal cord. Scale bar = 100μm.

I found that the RA catabolising enzyme, cyp26a, was weakly expressed in the unlesioned adult spinal cord in dorsal and ventral midline glia, spinal progenitors in dorso-lateral and ventro-lateral positions around the ventricle and in scattered neurons. After a lesion, mRNA levels of cyp26a were strongly increased in the lesioned spinal cord. Rostral to the lesion site, expression of cyp26a was confined to ventricular spinal progenitors and a population of cells in the vicinity of the ventricular zone, predominantly in the ventral half of the spinal cord. However, cyp26a was weakly expressed in the dorsal midline of the spinal cord. Caudally, cyp26a was expressed in ventricular progenitors in dorso-lateral and ventro-lateral domains and in cells near the vicinity of the ventricular zone, but predominantly in the ventral half of the spinal cord. It is also important to point out that although cyp26a is expressed in the ventral midline, occupied by *sonic hedgehog* expressing ependymo-radial glial cells (Reimer et al., 2009) in the unlesioned spinal cord, after a lesion these cells are negative for cyp26a expression (n = 3; Fig. 5.3).

Crabp2a, a cellular retinoic acid binding protein was not detectably expressed in the unlesioned spinal cord by in situ hybridisation. However, after a lesion, mRNA expression rostral to the lesion site was detected in one or two cells in the ventro-lateral domain of the spinal cord. The cells expressing crabp2a were located very close to the lesion site and not throughout the 750μm area of regeneration (n = 3; Fig. 5.3).

The upregulation of cyp26a and crabp2a after a lesion are consistent with published gene expressions. However, the retinoic acid receptor subunits (rarab, rxrγa, rxrγb) that were found to be upregulated by PCR in the lesioned spinal cord showed little or no detectable expression by in situ hybridisation (n = 3; Fig. 5.1 and 5.3). This could be due to the amount of mRNA transcripts that can be detected using this method. An alternative strategy would be to use antibodies against the RA receptor subunits to identify the pattern of protein expression in the lesioned spinal cord.

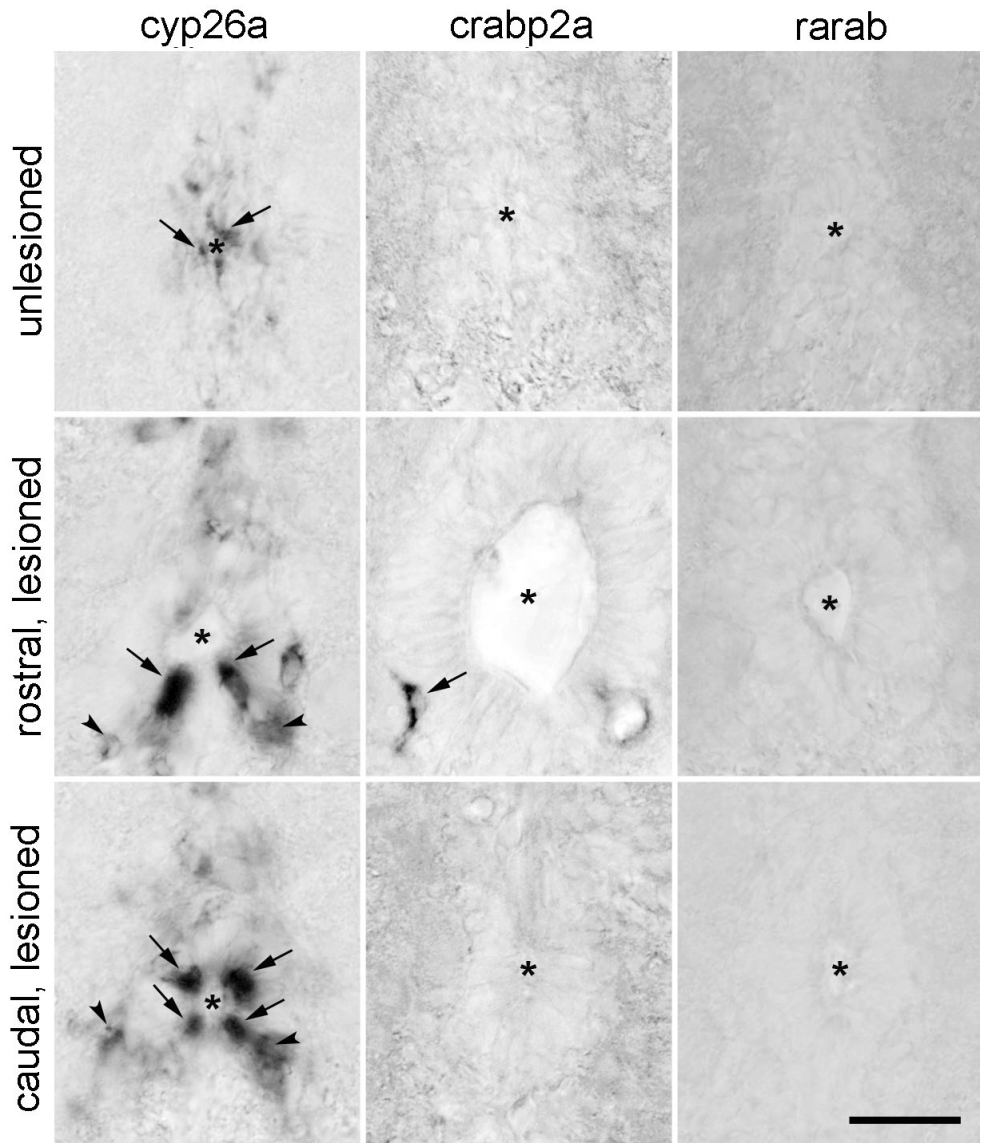

Fig. 5.3 Cyp26a and crabp2a are activated following a spinal lesion. Cross-sections of the unlesioned and lesioned spinal cord at 2wpl (dorsal is up). In situ hybridisation for candidate RA genes reveals that expression of cyp26a is enhanced in ventricular progenitors after a lesion compared to its expression in the unlesioned spinal cord. Furthermore, cyp26a was also detected in cells in the vicinity of the ventricular progenitor zone and predominantly in the ventral half of the spinal cord. Crabp2a was upregulated rostrally after a lesion in one or two cells (per section) in the ventro-lateral area of the spinal cord. Asterisks denote the ventricle of the spinal cord. RA receptor subunits (rarab, rxrγa, rxrγb) showed little or no mRNA expression as detected by the *in situ* hybridisation. Arrows mark ventricular cells with in situ signal and arrowheads denote expression in cells with no ventricular contact. Scale bar = 25μm.

5.2.2 Brain derived dopamine promotes the generation of spinal motor neurons during development and regeneration

5.2.2.1 Dopamine signals via D2-like receptors to promote motor neuron differentiation in vivo

An investigation of dopaminergic drugs revealed that those acting on D2-like receptors either promoted (agonists, reuptake inhibitor) or suppressed (antagonists) the generation of islet-1:GFP+ motor neurons (Fig. 5.4A and table 5.1 for all drugs) (Higashijima et al., 2000). In particular, the highly potent dopamine agonist R(-)-Propylnorapomorphine or NPA increased whereas the dopamine antagonist Haloperidol decreased the number of islet-1:GFP+ motor neurons in a dose dependent fashion (Fig. 5.4A). Importantly, the action of NPA was abolished by the D4 specific drug L-745870, providing strong evidence that NPA acts via the D4 receptor to enhance motor neuron generation in vivo (Fig. 5.4B). Using an HB9:GFP transgenic fish line which labels all motor neurons we observed a 38% increase and a 34% decrease in motor neuron numbers after NPA and L-745870 treatment respectively (Fig. 5.4C).

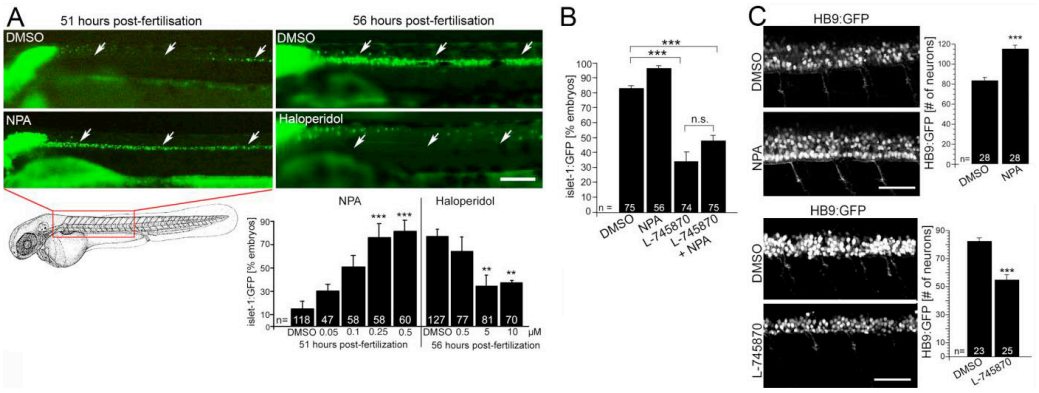

Fig. 5.4 Dopaminergics influence the number of spinal motor neurons. A: Lateral images of the trunk region of live Islet1:GFP transgenic zebrafish under a stereomicroscope are shown. NPA, a dopamine agonist (drug treatment 24 – 51hpf; ANOVA, ***P < 0.001 versus DMSO treated control) increases whereas Haloperidol, a dopamine antagonist (drug treatment 24 – 56hpf, ANOVA, **P < 0.01 versus DMSO) decreases Islet1:GFP+ motor neurons in the anterior spinal cord but in a ventral position. B: L-745870, a D4 specific antagonist attenuates the development of Islet1:GFP+ motor neurons, the effect of which cannot be rescued by NPA treatment (56hpf; ANOVA, ***P < 0.0001). C: Lateral views

of the trunk of HB9:GFP transgenic zebrafish at 33hpf are shown (drug treatment 24 to 33hpf). NPA increases by 38% while L-745870 significantly decreases by 35% the number of HB9:GFP+ motor neurons. Arrows denote Islet1:GFP+ motor neurons. Error bars represent SEM. Scale bar in A: 100μm: Scale bars in C = 50μm.

(Figure 5.4A was kindly provided by Dr. Michell M Reimer and figures 5.4B and C were provided by Anneliese Norris

Drug	**Action**	**Conc. (μM)**	**N =**	**P-value**	**% embryos promoted (+) or attenuated (-) compared to controls**
at 51 hpf (promotion)					
NPA	Agonist: D2, D3, D4 (D2<D4) (Lahti et al., 1993)	0.5	60	< 0.001	+67.2 ± 9.28
Pergolide	Agonist: D1, D2, D3,D4, D5 (Goldstein et al., 1980)	10.0	53	< 0.0001	+58.5 ± 12.7
R(-)-Apocodeine	Agonist: D1, D2, D3, D4, D5 (Seemann and Van Tol, 1994)	10.0	46	< 0.0001	+53.8 ± 15.90
GBR 12909	Reuptake inhibitor (Heikkila and Manzino, 1984)	10.0	46	= 0.0001	+49.2 ± 23.00
R(-)–2,10,11–Trihydroxy-N-propyl-noraporphine hydrobromide hydrate*	Agonist: D2, D3, D4 (Gao et al., 1990)	10.0	51	< 0.0001	+48.7 ± 7.22
Quinpirole	Agonist: D2, D3, D4 (Seemann and Van Tol, 1994)	5.0	60	< 0.001	+42.4 ± 2.78
Apomorphine	Agonist: D1-D5 (D1-like< D2-like, D2 < D4) (Lahti et al., 1993)	2.5	57	= 0.001	+29.4 ± 11.17

PD 168,077	Agonist: D4 (Glase et al., 1997)	10.0	68	= 0.003	+24.8 ± 6.86
Dopamine	Natural ligand	10	56	= 0.0057	+23.7 ± 12.38
SKF38393	Agonist:D1,D5 (Seemann and Van Tol, 1994)	10.0	59	n.s. (=0.1711)	-6.6 ± 1.75
at 56 hpf (attenuation)					
L-745870	Antagonist: D4 (Patel et al., 1997)	10.0	60	< 0.00001	-72.7 ± 4.15
L-741742	Antagonist: D4 (Rowley et al., 1996)	10.0	59	< 0.0001	-64.3 ± 7.42
Eticlopride	Antagonist: D2, D3, D4 (Kohler et al., 1986)	5.0	60	< 0.0001	-44.9 ± 2.50
Haloperidol	Antagonist: D2, D3, D4 (D2 > D4) (Lahti et al., 1993)	10.0	70	< 0.01	-39.8 ± 1.24

Table 5.1 Pharmacological profile of dopaminergics in promotion or attenuation of islet-1:GFP+ spinal motor neuron development (kindly provided by Ms. Anneliese Norris).

5.2.2.2 Diencephalic TH1+ descending projection axons promotes motor neuron generation in the developing spinal cord

Tyrosine hydroxylase (TH) enzymes are rate-limiting for dopamine synthesis. Immunohistochemistry for TH1 reveals TH1+ cells in the diencephalon that send out descending projection axons. These axons innervate the brainstem at 24hpf but progressively grow longer from 33hpf to 56hpf into the spinal cord (Fig. 5.5A,B) (McLean and Fetcho, 2004a). TH1+ axons grew in close proximity to Olig2:GFP+ motor neurons and their progenitors in the ventral spinal cord (Fig. 5.5C) (Shin et al., 2003). To determine whether endogenous dopamine is necessary for motor neuron generation we used an anti-sense morpholino oligonucleotide to

knockdown th1. The morpholino abolished all TH1 immunoreactivity in 33hpf embryos (Fig. 5.5D) and also reduced the numbers of HB9:GFP+ motor neurons by 32% (Fig. 5.5E,F). The growth of Islet1:GFP+ motor neurons was also significantly attenuated by morpholino treatment at 56hpf (Fig. 5.5G). However, treatment of TH1 morphants with the dopamine agonist NPA rescued the motor neuron deficit, suggesting that the drug compensated for the reduced dopamine synthesis. Thus, brain-derived TH1+ axons are the only detectable source of dopamine in the embryonic spinal cord and are at the right time place to regulate the differentiation of motor neurons (Fig. 5.5H).

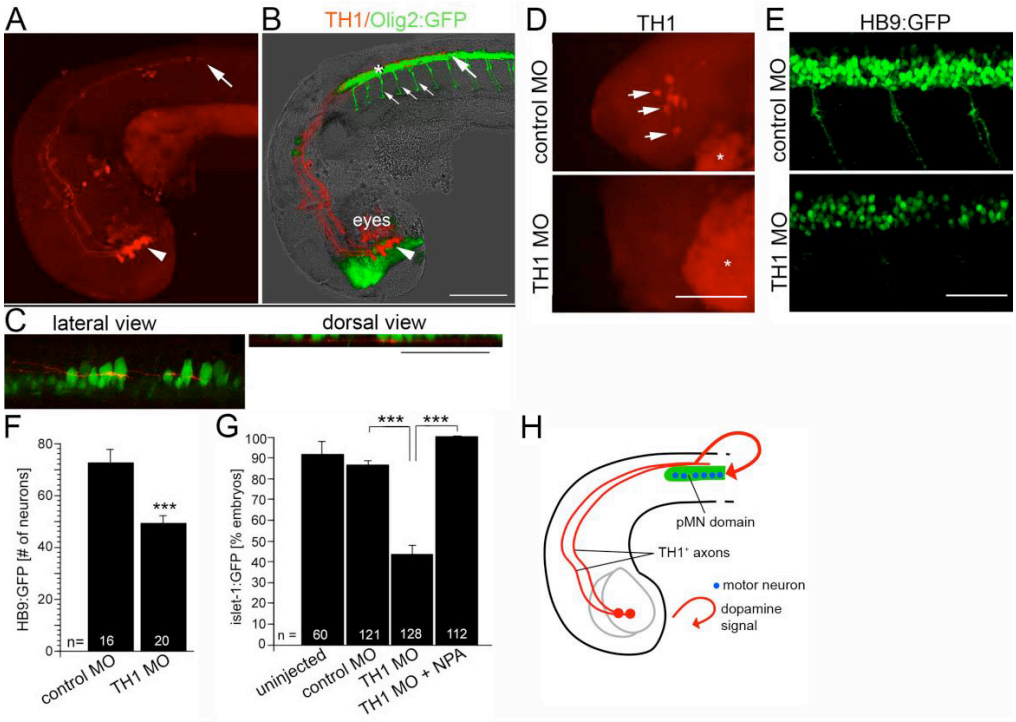

Fig. 5.5 Descending dopaminergic axons promotes the generation of motor neurons during development. A-C: Lateral view showing diencephalic TH1+ dopaminergic neurons and axonal growth cones of their spinal projections that grow in close proximity to Olig2:GFP+ motor neuron progenitors as revealed in higher magnifications. The projection axons reached the brainstem at 24hpf (n=7) and reached they reached somite 7± 0.2 (n=8) by 33hpf and somite 18 ± 2.4 (n=10) by 56hpf. Arrowheads indicate TH1+ diencephalic neurons; large arrows mark growth cones; small arrows point to motor axons. D: Side views of embryonic heads showing knockdown of TH1 with a morpholino antisense oligonucleotide that leads to a loss in TH1+ positive diencephalic neurons. Arrows indicate TH1 immunoreactivity; asterisks mark autofluorescence of the yolk sac.

E-G: Knockdown of TH1 leads to a significant loss of HB9:GFP+ motor neurons (at 33hpf; ***P = 0.001) and attenuation of Islet1:GFP+ motor neuron development which is completely rescued by NPA treatment (ANOVA, ***P < 0.0001). H: Schematic showing the spatial relationship between TH1+ diencephalic dopaminergic neurons, their axonal projections and the spinal pMN domain. Error bars indicate SEM. Scale bars in A, B = 200μm, C = 50μm, D = 100μm; E = 50μm.

(Figure 5.5 was kindly provided by Ms. Anneliese Norris).

5.2.2.3 The dopamine receptor D4a is necessary to generate the correct numbers of motor neurons

Next, we addressed the role of D2-like receptors in the spinal cord, as indicated by the pharmacological profiling (see above). In particular, we used two independent splice site directed morpholino anti-sense oligonucleotides to knockdown D4a expression in the embryonic spinal cord. Treatment with the morpholino oligonucleotides abrogated D4a expression (Fig. 5.6A) and decreased the number of HB9:GFP+ motor neurons by 49% (1mM MO1), 47% (1mM MO2) and 40% (0.5mM MO1 + 0.5mM MO2) at 33hpf (Fig. 5.6C). The appearance of islet1:GFP+ motor neurons was strongly reduced in D4a morphant embryos. Importantly, treatment with the agonist NPA did not rescue the reduction of motor neurons in the morphants (Fig. 5.6B). This is contrary to the effect of knockdown of TH1, where NPA rescued the motor neuron deficit (Fig. 5.5G). In summary, our observations indicate that the D4a receptor is vital to mediate dopamine's action on motor neuron generation. Dopaminergic axons innervate the embryonic spinal cord later than 24hpf and thus cannot influence early motor neuron generation which starts at 9hpf (Myers et al., 1986). Thus, endogenous dopamine signalling does not regulate motor neuron generation before TH1+ dopaminergic projections arrive in the spinal cord.

In order to determine whether dopamine acts on progenitor cell proliferation we labeled for the mitotic marker pH3 in the Olig2:GFP+ progenitor zone after treatment with NPA in the same time window as the elicited increase in motor neuron generation (24 to 33hpf). A 73% increase in the number of Olig2:GFP+/pH3+ mitotic progenitors was observed after NPA treatment (Fig.

5.6D). Long-range dopamine signalling could interact with local signalling networks to regulate spinal neurogenesis. The hedgehog signalling pathway is essential for motor neuron differentiation (Fuccillo et al., 2006). Thus, we analyzed increased hedgehog signalling as a possible downstream effect of D4a activation. Expression levels of patched1, the hedgehog target gene were significantly increased following NPA treatment. Cyclopamine, an antagonist of the hedgehog receptor smoothened strongly reduced expression levels of patched1 but NPA application in the same embryos had the opposite effect and increased the levels of patched1. This suggests that dopamine could substitute for hedgehog signalling (Fig. 5.6E).

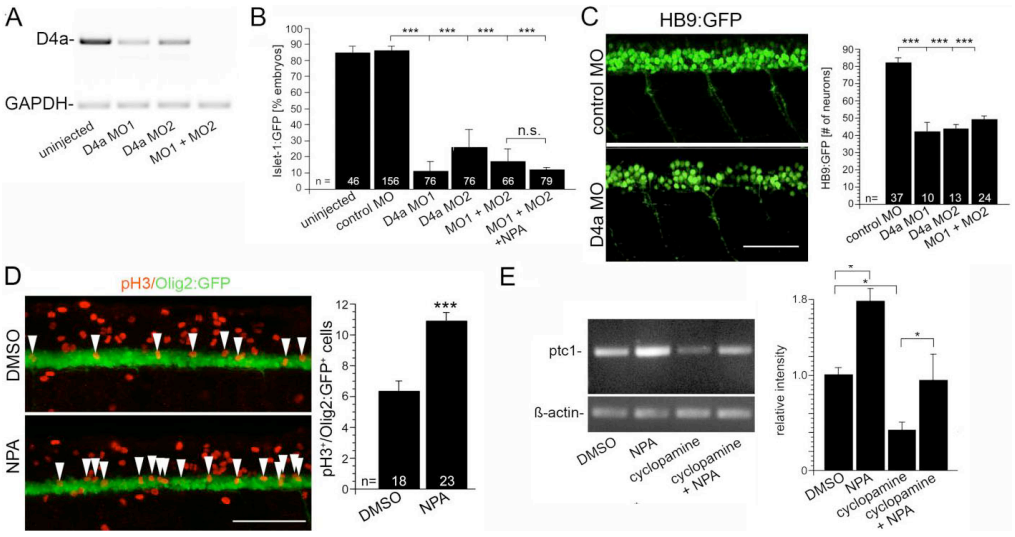

Fig. 5.6 Dopamine receptor D4a is required to mediate its action on spinal motor neurogenesis. A: PCR analysis shows that D4a morpholino knockdown is efficient. B-C: D4a knockdown attenuates Islet1:GFP+ motor neuron development which could not be rescued by NPA (ANOVA, ***P < 0.0001) and reduces the number of HB9:GFP+ motor neurons (ANOVA, ***P < 0.0001). D: Treatment with NPA increases the proliferation of pMN progenitors. Lateral trunk views are shown. Olig2:GFP embryos reveal an increased number of pH3-positive cells in the pMN zone at 33hpf (***P < 0.0001). Arrowheads indicate double labeled Olig2:GFP/pH3+ proliferative progenitor cells. E: Dopamine acts on the hedgehog pathway as detected by PCR analysis. There is a significant upregulation of the hedgehog target gene patched1 in NPA treated embryos (ANOVA, P= 0.013) and the rescue of ptch1 levels in NPA/cyclopamine double treated embryos (incubation from 24 to 51hpf, ANOVA, P = 0.025). Error bars represent SEM. Scale bars = 50μm. (Figures 5.6B, C and D were kindly provided by Ms. Anneliese Norris and figure 5.6 E was provided by Dr. Zhen Zhong).

5.2.2.4 Endogenous brain-derived dopamine promotes the regeneration of motor neurons after spinal cord lesion

It has been previously shown that developmental signals such as hedgehog signalling can be recruited during motor neuron regeneration after a spinal lesion in adult zebrafish (Reimer et al., 2009). We thus tested whether dopamine signalling could also promote motor neuron regeneration. A complete transection of the spinal cord elicits the generation of new motor neurons rostral and caudal close to the lesion site which peaks at 14dpl (Reimer et al., 2008). We analyzed the availability of endogenous TH1+ axons at that time point. The density of TH1+ axons had increased to 42% rostral to the lesion site (Kuscha et al., 2011) thus indicating axonal sprouting of severed descending axons (Fig. 5.7A). Some TH1+ axons were located in close proximity to Olig2:GFP+ ependymo-radial glial motor neuron progenitors at the central canal (Fig. 5.7B) (Reimer et al., 2008). Very few TH1+ profiles were detected caudal to the lesion site mainly because these axons had not grown beyond the lesion site at 14dpl (Kuscha et al., 2011). This indicates that similar to the developing spinal cord, dopaminergic innervation in the adult spinal cord is also exclusively derived from the brain.

In order to identify which receptor/s dopamine mediates its action through to generate motor neurons during spinal cord regeneration, I used plasmids for D2a, D2l, D3 and D4a (Boehmler et al., 2004; Boehmler et al., 2007) and cloned the D2b and D4b receptors into expression vectors to synthesize antisense probes. Using *in situ* hybridization I initially validated the probes in the unlesioned brain (n = 3 for each gene; Fig. 5.8). mRNA transcripts for the dopamine receptor d2a were found to be expressed in cells in the tectal commissure (Ctec) of the rostral optic tectum. Drd2b was expressed exclusively in cells at the periphery of the ventral telencephalic area whereas drd2l was found to be expressed in the periventricular ventral hypothalamus in the diencephalon. Cells in the crossed tecto-bulbaris tract (TTBc) and the ventral rhombencephalic commissure (Cven) expressed drd3 specific mRNA. The dopamine receptors d4a and d4b were expressed in the

periventricular nucleus of the posterior tuberculum (TPp) in close proximity to the diencephalic ventricle and the corpus cerebeli (CCe) in the rostal part of the optic tectum respectively. The in situ expression patterns for the dopamine receptors were consistent and in accordance with published results. Thus, confirming the accuracy and quality of the mRNA probes.

Next, I determined the expression patterns of these receptors in the unlesioned and lesioned spinal cord. Since motor neuron regeneration after a spinal cord lesion peaks at 2wpl (Reimer et al., 2008), *in situ* hybridization for the dopamine receptors in the lesioned spinal cord was performed at that time point. The D4a receptor was strongly upregulated in the entire ventricular zone of the rostral spinal cord close to the lesion site but not caudal to it. Other D2-like receptors were not conspicuously expressed in the ependymal zone For instance; the D2a receptor was expressed in mature neurons at the same expression level in the unlesioned and lesioned spinal cord (n = 3 for each gene; Fig. 5.9). The observation that the D4a receptor was not detectably upregulated caudal to the spinal lesion suggests that a signal from the descending axons potentially the dopaminergic axons themselves is needed for for increased receptor expression. Hence, rostrally signal (TH1+ axons) and receptor (D4a mRNA) are present for dopamine to mediate its action on motor neuron regeneration (Fig. 5.7A) whereas; caudally regeneration occurs without dopamine signaling due to a lack of signal and receptor.

Consistent with these rostro-caudal assymetries in the presence of dopamine signal and receptor, 50% more motor neurons identified by HB9:GFP+ transgene expression or intense HB9 immunoreactivity are generated rostrally in untreated animals at 14dpl (Fig. 5.7C). Repeated intraperitoneal treatment with the dopamine agonist NPA (3, 6 and 9dpl – analysis at 14dpl) increased the number of motor neurons by 50% rostral to the lesion site, but was ineffective caudally (Fig. 5.7C). To determine if NPA acts on progenitor cell proliferation during spinal cord regeneration, we applied BrdU in NPA treated Olig2:GFP transgenic fish. We

found a 53% increase in the number of Olig2:GFP+ motor neuron progenitor cells only rostrally, thus indicating that NPA acts on progenitor cell proliferation (Fig. 5.7D).

Regenerative signals from descending axons do not reach the progenitor cells, caudal to the lesion site because of their slow or lacking regrowth. Therefore, we wanted to determine if a dopamine agonist could substitute for the lack of dopamine signaling caudally. To mimic the dopaminergic innervation we used repeated injections of NPA (injections at 3, 5, 6, 7, 8 and 9 dpl – analysis at 14dpl). A 79% increase in the number of HB9+ motor neurons was observed caudal to the lesion site (Fig. 5.7E). Consequently, an increase in the expression levels of D4a and patched1 by NPA treatment was observed caudal to the spinal lesion (Fig. 5.7E). In summary a continous dopamine signal substituted by NPA can activate D4a expression and lead to increased motor neuron regeneration in the absence of dopaminergic axons. Rostrally, recurrent injections did not have a positive effect on the number of motor neurons or on gene expression. A possible overstimulation of the pathway by the combination of injected and endogenous signal occurs there.

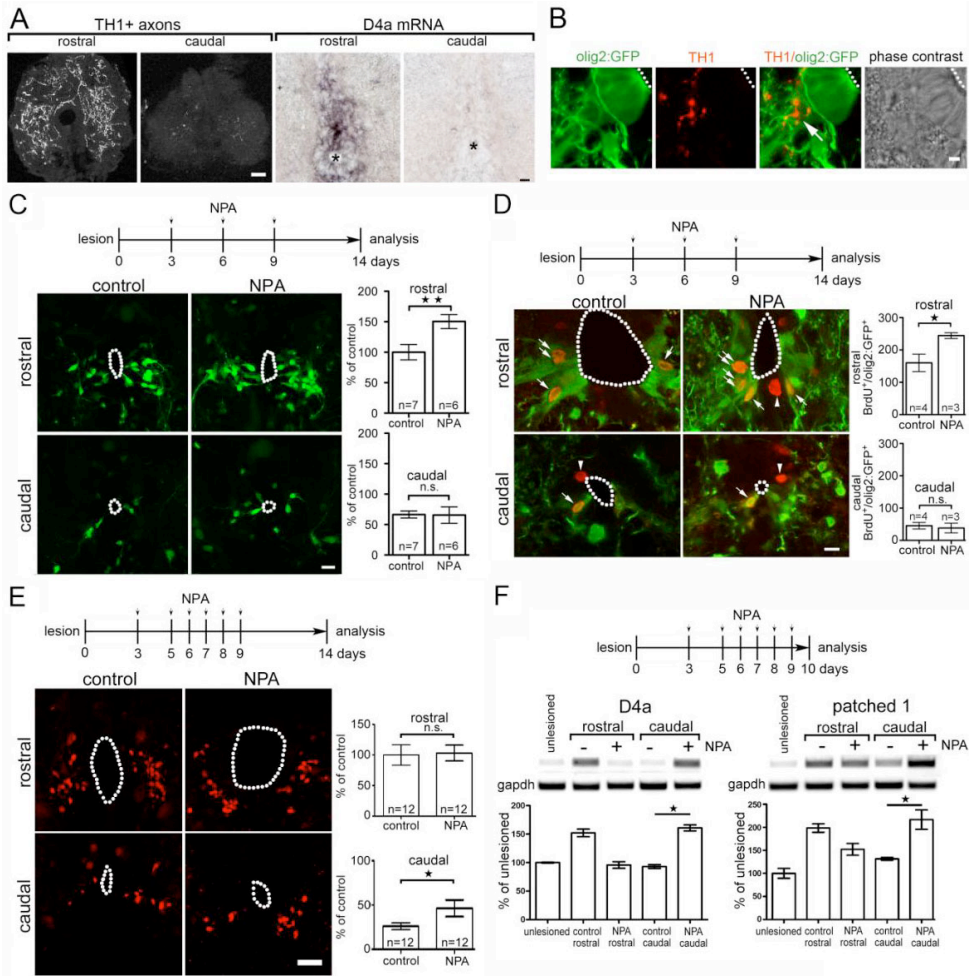

Fig. 5.7 Endogenous dopamine promotes motor neuron regeneration after a spinal cord lesion. Spinal cross-sections at 14dpl are shown (dorsal is up; central canal is outlined by dots). Timelines for experimental manipulations are shown. A: Increased TH1 immunoreactivity and D4a receptor expression is detected only rostral to the lesion site. B: High magnification images reveals apposition denoted by arrows of TH1+ axons with Olig2:GFP+ pMN progenitor cells. C: Application of the dopamine agonist, NPA at low frequency increases the number of HB9:GFP+ motor neurons only rostral to the lesion site (**P < 0.01). D: NPA increases the number of Olig2:GFP+ pMN progenitor cells only rostral to the lesion site. Arrows indicate Olig2:GFP+ cells (green) with its nucleus labeled with BrdU (red); arrowheads mark BrdU+ ventricular cells that are Olig2:GFP- (*P = 0.0268; one-tailed Mann-Whitney U test). E: NPA application at high frequency significantly increases the number of motor neurons only caudal to the lesion site (*P < 0.05, one-sided). F: High frequency NPA injections increase D4a receptor and patched1 expression caudal to the spinal lesion. Quantitative densitometric analyses are shown (*P < 0.05; Kruskal-Wallis with Dunn's post-hoc test). Error bars represent SEM. Scale bars in A = 40µm (left) and 20µm (right); B = 5µm; C,E = 15µm; D = 10µm. (Figures 5.7A showing TH1+ immunostaining, 5.7B and 5.7D were provided by Dr. Veronika Kuscha. Figures 5.7C, E and F were provided by Dr. Michell M Reimer).

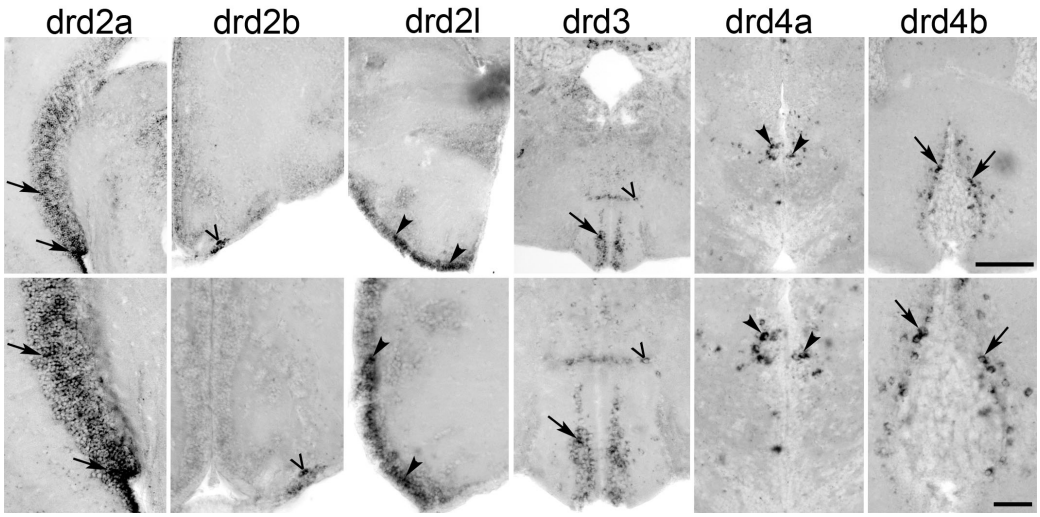

Fig 5.8 Dopamine receptors are expressed in the adult zebrafish brain. Crossections of different regions of the brain are shown. Dorsal is up. Images in the top panel were taken using 10X objective while images in the bottom panel are high magnification views of the same. Drd2a was expressed in cells in the Ctec in the rostral optic tectum (denoted by arrows). mRNA transcripts for drd2b were found in ventral telencephalic cells (marked by open arrowheads). In situ signal for drd2l was detected in the periventricular ventral hypothalamus in the diencephalon (indicated by arrowheads) whereas cells in the TTBc (depicted by arrows) and the Cven (marked by open arrows) expressed drd3 specific mRNA. The dopamine receptors d4a and d4b were expressed in the TPp lining the diencephalic ventricle (denoted by arrowheads) and the CCe (shown by arrows) in the rostal part of the optic tectum respectively. Scale bars = 400μm (top panel), 50μm (bottom panel).

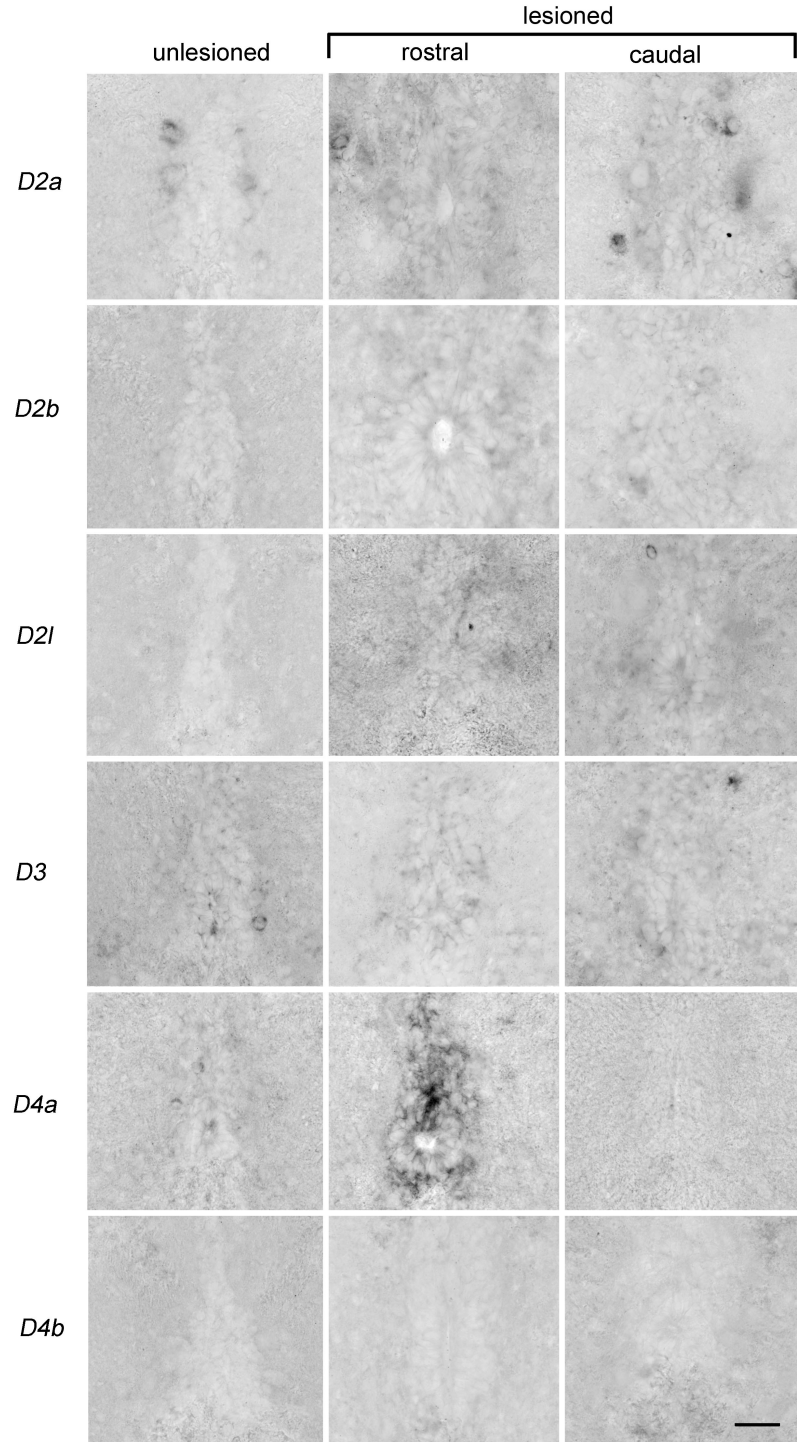

Fig. 5.9. Dopamine likely signals via the D4a receptor. Cross sections of the adult unlesioned and lesioned spinal cord at 2wpl are shown. Dorsal is up. In situ hybridisation reveals that the D4a receptor alone and not the other D2-like receptors is upregulated in all ventricular spinal progenitor cells rostral to the lesion site but not caudal to it. D2a was expressed in mature neurons in the unlesioned and lesioned spinal cord. Scale bar = 25μm

5.3 Discussion

5.3.1 Cyp26a and Crabp2a are upregulated after a lesion in ventricular progenitors

We demonstrate here using in situ hybridisation that signalling components of the RA pathway, namely the downstream genes cyp26a and crabp2a, are upregulated in the adult lesioned spinal cord. This is consistent with published work showing that cyp26a and crabp2a are upregulated after a lesion as assessed by PCR (Reimer et al., 2009). Interestingly, cyp26a and crabp2a are differentially upregulated in the rostral and caudal spinal cord. The number of cells expressing the cyp26a mRNA was higher in the caudal spinal cord than the rostral spinal cord. Crabp2a is expressed in few cells close to the ventricular zone in the rostral half of the lesioned spinal cord but not caudal to it.

Olig2+ ependymo-radial glia i.e the ventricular spinal progenitor cells in the ventro-lateral domain are the bonafide motor neuron progenitors (Reimer et al., 2009). We postulate that cells expressing the cyp26a mRNA in the ventro-lateral domain of the progenitor zone are likely to be those motor neuron progenitors. In addition, cells in the ventro-lateral region in the adult lesioned spinal cord are HB9+ new born motor neurons (Reimer et al., 2008). Thus, cells in the vicinity near the progenitor zone that express either cyp26a or crabp2a are probable differentiating neurons. To confirm our hypothesis, it would be informative to test the expression of cyp26a in ependymo-radial glial cells in olig2:GFP transgenic animals and crabp2a in differentiating motor neurons in HB9:GFP transgenic animals.

In the developing spinal cord, RA specifies the p0 and p1 domains in the dorsal part of the ventral neural tube along the dorso-ventral axis (Pierani et al., 1999). In the chick spinal cord, treatment with a shh agonist and RA enhances the generation of Olig2+ motor neuron progenitors from the pMN domain. Blockade of RA signalling impairs the progression of Pax6+ Nkx6+ ventral progenitor cells to an

Olig2+ motor neuron progenitor state. Furthermore, retinoid receptor activation is required for the Hh induction of Olig2 and motor neuron generation *in vivo* (Novitch et al., 2003). A recent study in zebrafish that lack the maternal and zygotic Hh signaling transducer smoothened (MZsmo mutants) showed that primary motor neurons still persisted in its absence. Furthermore, the primary motor neurons required RA and basal Gli activity where RA modulated Gli function in a Hh ligand-independent manner (Mich and Chen, 2011). RA has also been shown to influence the generation of V0, V1, and V2 interneurons, mediated through neurogenin2 (Ngn2) (Novitch et al., 2003). Interestingly, RA was shown to be required for the correct number of spinal neurons to form (England et al., 2011).

Given that developmental signals such as shh and RA that act locally are re-activated after a lesion, we hypothesize that RA may act autonomously or in concert with shh to generate new motor neurons. Moreover, since cyp26a is expressed in ventricular progenitor cells in the ventro-lateral and dorso-lateral regions of the spinal cord, we hypothesize that these cells may contribute to motor neuron and interneuron generation. To examine the importance of RA for the numbers of motor neurons or interneurons, we could inhibit the pathway using DEAB (4-(Diethylamino)-benzaldehyde) a competitive, reversible inhibitor of retinaldehyde dehydrogenases which is active in zebrafish (Begemann et al., 2004) or BMS493 that antagonizes the activity of all RARs, also known to function in zebrafish (Fig. 5.10; (Stafford and Prince, 2002). Numbers of HB9 positive motor neurons as detected by immunohistochemistry would be the read-out. These manipulations would give us some insight into the mode of action of RA on spinal neurogenesis.

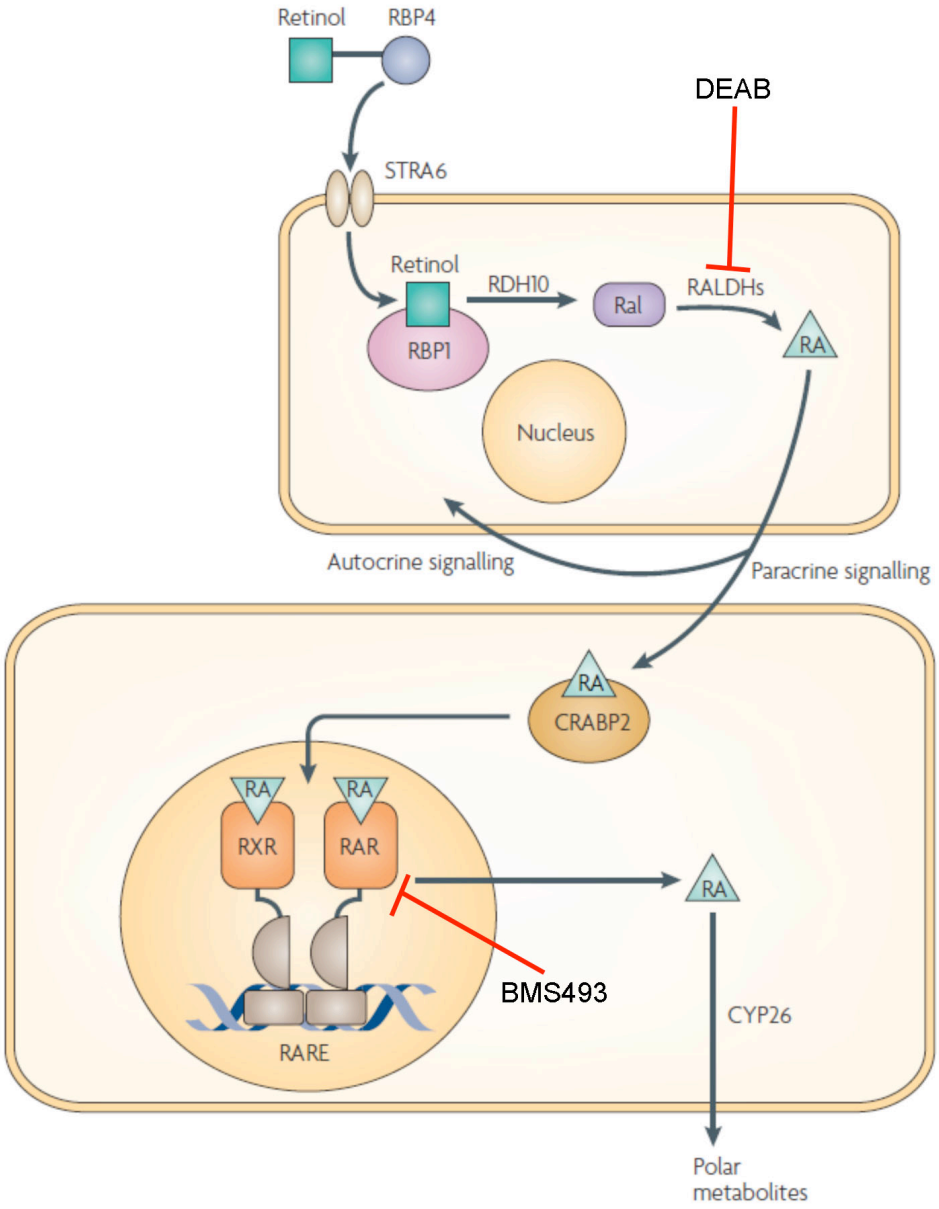

Fig 5.10. Schematic of RA pathway with pharmacological tools. RBP4 = retinol-binding protein 4 plasma, RBP1 = retinol-binding protein 1, cellular, RDH10 = retinol dehydrogenase 10, RALDH = retinaldehyde dehydrogenases, RA = all-trans retinoic acid, CRABP2 = cellular retinoic-acid-binding protein 2, RXR = retinoic X receptor, RAR = RA receptor, RARE = retinoic acid-response element and CYP26 = Cytochrome P450 26. Image modified from (Maden, 2007).

5.3.2 Dopamine signals via D4a to influence the number of motor neurons

We have demonstrated here that dopamine is likely to signal via the D4a receptor to influence the numbers of motor neurons during development and regeneration of the zebrafish spinal cord. Furthermore, our findings reveal dopamine acting on hedgehog signalling as a first signal by which descending axons regulate neurogenesis in the ventral spinal cord.

Dopamine in the developing spinal cord

Descending dopaminergic axons are among the first to innervate the spinal cord during development (McLean and Fetcho, 2004a, b) with no detectable spinal intrinsic dopamine. In chick explant cultures, manipulations of hedgehog levels show balanced changes in the number of V2 interneurons and motor neuron numbers (Karunaratne et al., 2002). Since dopamine acts on hedgehog signalling, it would be tempting to assume that manipulating dopamine levels could affect the number and type of spinal neurons. Thus, it would be informative for future studies to determine if dopamine exerts any effects on V2 interneurons.

Dopamine in the adult regenerating spinal cord

Treatment with a dopamine agonist following a spinal lesion increases the number of newly generated motor neurons rostral to the lesion site but not caudal to it. Thus, the dopamine signal is sufficient for increased neurogenesis. Importantly, dopamine regulates expression of its own receptor on spinal progenitors. This is supported by the observation that during unmanipulated regeneration, expression of the d4a receptor is higher rostral (sprouting of TH1+ axons) than caudal (TH1+ axons absent) to the lesion site (Fig. 5.11). Furthermore, daily dopamine agonist injections increase d4a expression also caudal to the lesion site.

Hh signalling is necessary for motor neuron regeneration (Reimer et al., 2008). Injection of a dopamine agonist increased the expression of patched1 (ptc1), a downstream target gene of shh signalling both rostral and caudal to the lesion site. This suggests that dopamine signalling converges on the Hh signalling pathway via ptc1 in motor neuron progenitors. These results demonstrate an important asymmetry between the rostral and caudal parts of the lesioned spinal cord. We show for the first time that descending axons secrete dopamine that acts on ptc1 in pMN progenitors and influences the generation of motor neurons.

In the injured rat spinal cord, there are higher numbers of proliferating cells rostral to the lesion site than caudal to it (McTigue et al., 2001). This is similar to our observation in zebrafish where more motor neurons regenerate only rostrally. It is tempting to speculate that dopamine could influence the generation of motor neurons in the mammalian spinal cord. In the mammalian cerebral cortex, it has been suggested that dopamine acts directly on progenitor cells (Hoglinger et al., 2004). Following a lesion, the altered activity of the spinal circuitry in response to dopamine signalling could stimulate the generation of motor neurons (Spitzer, 2002). Mammalian ventricular ependymal cells, similar to those seen in the zebrafish proliferate after a lesion (Meletis et al., 2008). Although these cells have a potential to generate neurons when transplated into a neurogenic area of the brain (Shihabuddin et al., 2000), in situ they make predominantly glial cells that contribute to scar formation (Barnabé-Heider et al., 2010). It is therefore important to identify mechanisms that would bias progenitor cells to a neurogenic fate in the mammalian spinal cord. Altering the endogenous levels of dopamine could be the answer to influencing the generation of motor neurons in the spinal cord.

Subsequent interventions could be beneficial caudal to the lesion site, an area that is devoid of any dopaminergic input because descending axons do not regenerate in mammals. In motor neuron diseases such as amyotrophic lateral sclerosis (ALS), motor neurons are progressively lost and never replaced. In a mouse model of

ALS, increased proliferation of ventricular progenitors and limited neurogenesis has been observed (Chi et al., 2006). Although, there are many factors that contribute to the reduced regenerative potential in the mammalian spinal cord, maybe dopamine could be used to harness the endogenous repair mechanisms of spinal progenitors (Boulis et al., 2011).

In summary, the RA and dopamine signalling pathways acting autonomously or in concert with Hh may be involved in the generation of motor neurons in the adult lesioned spinal cord.

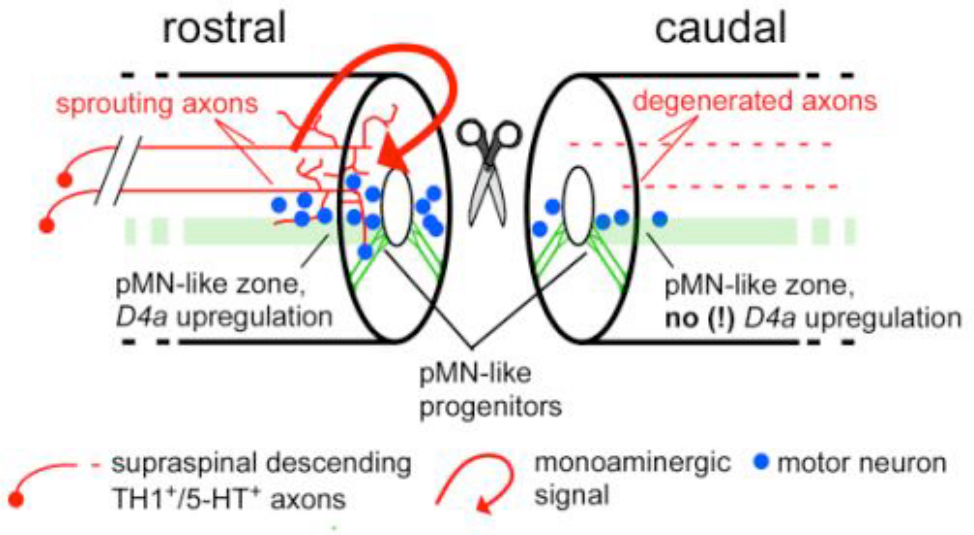

Fig 5.11. Schematic of the lesioned spinal cord in a lateral view. TH1+ axons are present and sprout only rostral to the lesion site, this coincides with more motor neurons and upregulation of the D4a receptor at 2 weeks post-lesion. In contrast, the caudal spinal cord has degenerated TH1+ axons and less numbers of motor neurons with no upregulation of D4a. Shh signalling and proliferation is increased both rostral and caudal to the spinal lesion site (Reimer et al., 2009).

Conclusions

In the mammalian spinal cord, an inhibitory environment around the lesion site, the glial scar, and possibly activation of the Notch pathway result in the low regenerative potential after injury. It is difficult to test molecular mechanisms required for successful spinal cord regeneration in essentially a non-regenerating system. However, zebrafish in contrast to mammals, can regenerate severed axons and replace lost neurons after a complete spinal cord transection (Becker et al., 2004; Reimer et al., 2008). I have therefore used the adult zebrafish to examine the role of signalling pathways during spinal cord regeneration. The insights we gain from these studies will inform better therapeutic interventions to promote regeneration in the mammalian spinal cord. It has been previously shown that Olig2 positive ependymo-radial glia in the ventricular progenitor zone is the spinal progenitor cells that proliferate after a lesion and generate motor neurons. Furthermore, shh secreted from the floor plate is necessary for motor neuron generation rostral and caudal to the lesion site (Reimer et al., 2009).

6.1 Notch signalling controls the generation of motor neurons in the lesioned spinal cord of adult zebrafish

The Notch signalling pathway has been implicated in being responsible for low regenerative potential of spinal progenitors to generate motor neurons (Yamamoto et al., 2001). In chapter 3 of this dissertation, I investigated the role of Notch signalling in the regenerating spinal cord of the adult zebrafish. I found that Notch activation in the lesioned spinal cord attenuated the generation of motor neurons and subsequent proliferation. Remarkably, this is similar to the effect of Notch in the injured mammalian spinal cord (Yamamoto et al., 2001). If we blocked Notch activity using DAPT, an inhibitor for the γ-secretase complex, I observed an increase in progenitor proliferation and the number of motor neurons generated. Thus, Notch acts as a negative regulator of progenitor proliferation and motor

neuron regeneration also in the adult zebrafish spinal cord. Therefore, like in the mammalian spinal cord, high Notch levels may contribute to limited regenerative potential.

6.2 The role of Notch in neurogenesis in the intact adult zebrafish retina

In chapter 4, I investigated the role of Notch in the adult intact zebrafish retina. This study was prompted by an incidental find, while using the same manipulation to activate Notch in the lesioned spinal cord. I found that while Notch activity attenuated proliferation and motor neuron generation in the lesioned spinal cord, it induced a massive increase in eye size and proliferation of presumptive Müller glia in the INL of the central retina. Proliferation in the normal central retina is sparse, however we speculate that if Notch induces Müller glia to proliferate it could generate all types of retinal neurons. However, it is important to confirm that the proliferative cells in the INL are indeed Müller glia. Taken together, chapters 3 and 4 explore the role of Notch in different adult progenitor pools revealing that it may play very different roles in different neurogenic niches.

6.3 The role of dopamine and RA during spinal cord regeneration

Investigating the role of additional signalling pathways that are necessary for motor neuron regeneration, in chapter 5, we show for the first time that dopamine secreted by supraspinal descending axons signals via the dopamine receptor D4a on spinal progenitor cells to influence motor neuron differentiaton during development and regeneration. Furthermore, dopamine likely acts on hedgehog signalling to regulate the numbers of motor neurons generated. Treatment with a dopamine agonist results in twice as many motor neurons rostral to the lesion site. It is important to note that dopamine regulates its own receptor. In the untreated lesioned spinal cord the expression of d4a was increased rostral (TH1+ axonal

sprouting) than the expression caudally (no TH1+ axons). Consequently, daily injections of the agonist led to an increase in D4a expression caudal to the lesion site.

To determine the role of RA during motor neuron regeneration, I used in situ hybridisation to assess gene expression patterns for RA genes that have previously been found to be upregulated after a lesion by PCR (Reimer et al., 2009). I found that the downstream genes of the RA pathway cyp26a and crabp2a were expressed in ventricular progenitor cells and in the region close to vicinity of the pMN progenitor domain respectively. This suggests that RA may be involved in lesion-induced motor neuron generation. Nevertheless, functional studies need to be performed to help us elucidate if RA is necessary during spinal cord regeneration.

6.4 Putative interactions between signalling pathways in the lesioned spinal cord

Given what we know about the role of Hh during spinal cord regeneration (Reimer et al., 2009) and the insights gained about the roles of the Notch, dopamine and RA signalling pathways in this thesis I would like to discuss their potential interactions with each other and how this might relate to motor neuron regeneration (Fig. 6.1).

In the lesioned spinal cord, Hh signalling that is essential for motor neuron regeneration is expressed rostral and caudal to the lesion site. The same is true for Notch signalling as seen in chapter 3 bearing in mind that Notch serves as a negative regulator of neurogenesis. As seen in chapter 5, dopamine from descending axons act via the D4a receptor and converge on the Hh pathway to influence motor neuron differentiation in the rostral spinal cord. Furthermore, the RA binding receptor, crabp2a is expressed in the pMN-like zone in the rostral spinal cord while the RA catabolizing enzyme, cyp26a is expressed at a higher level in the caudal spinal cord.

From these observations, it becomes clear that dopamine serves as a master regulator of motor neuron differentiation in the spinal cord as it regulates Hh signalling to influence motor neuron regeneration. Notch signalling is anti-neurogenic and serves to control the timing of cell birth while maintaining the pool of spinal progenitors. In the developing spinal cord RA is required for the specification of motor neurons where retinoid acid receptor activation is necessary for the Hh induction of Olig2 and motor neuron generation *in vivo* (Novitch et al., 2003). Indeed crabp2a is upregulated in the pMN-like zone in the rostral spinal cord. More than half the number of motor neurons as identified by HB9:GFP+ transgene expression or intense HB9 immunoreactivity are generated rostrally in untreated animals at 14dpl. It is tempting to speculate that rostral to the lesion site, dopamine and RA signalling converge on the Hh pathway to influence the generation of motor neurons, the process of which is regulated by the Notch pathway. It is plausible that the dopamine/Hh signal could over-ride the Notch signalling to promote regeneration and it may why twice as many motor neurons at generated rostrally. Therefore, one might hypothesize that increasing dopamine signalling should in turn increase Hh activity and subsequently decrease Notch signalling leading to an increase in progenitor cell proliferation and motor neurons. Conversely, increasing Notch signalling should decrease both dopamine and Hh signalling and result in a reduction of proliferation and fewer motor neurons. However, these hypotheses would need to tested in the future.

In the caudal spinal cord, regeneration takes place in the absence of dopamine signal, as the growth of descending dopaminergic axons is slow and lacking regrowth. However, the role of Hh signalling in positively influencing progenitor cell proliferation and generation of motor neurons is probably negatively regulated by active Notch signalling. Interestingly, the RA catabolizing enzyme, cyp26a is expressed in spinal progenitor cells. Taken together, this may be one of the reasons why the number of motor neurons are reduced compared to the rostral spinal cord. It is possible that another signalling pathway acting in concert with Hh contributes

to proliferation in the caudal cord. In motor neuron disease, there is significant loss of lower motor neurons and therefore it would be very useful to identify signalling pathways that can be manipulated to enhance regeneration in the caudal spinal cord. An alternative approach would be to increase dopamine signal caudally so as to enhance neurogenesis as done in chapter 5.

It is important to note that the role of RA plays the regenerating spinal cord mentioned above has been used in purely speculative manner and functional experiments need to done to elucidate its mode of action during spinal cord regeneration. It is clear that while for each signaling pathway, distinct roles can be defined; none of them work in isolation. The regulatory interactions between the Hh, Notch, dopamine and RA pathways need to be detailed so as to define the essential elements of successful neuronal regeneration.

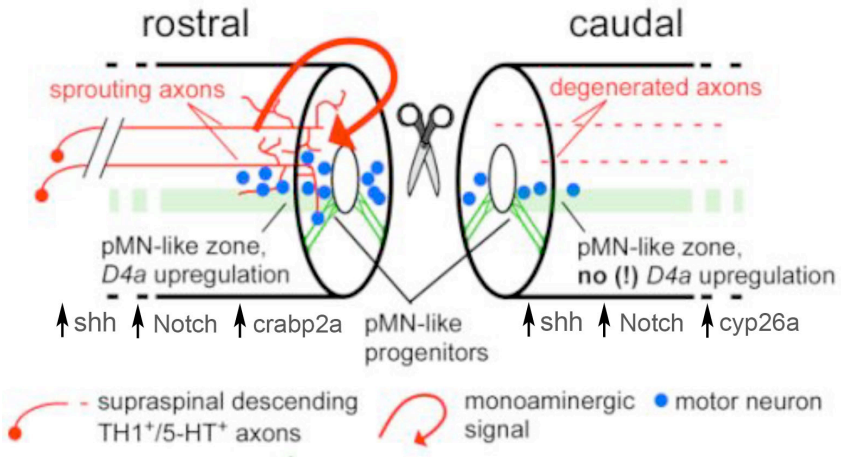

Fig 6.1. Schematic of the lesioned spinal cord in a lateral view with an overview of expression of different signalling pathways during motor neuron regeneration. Hh signalling, progenitor proliferation (Reimer et al., 2009) and the Notch pathway is increased both rostral and caudal to the spinal lesion site. TH1+ axons are present and sprout only rostral to the lesion site coinciding with an increase in more motor neurons and upregulation of the D4a receptor at 2 weeks post-lesion. In contrast, the caudal spinal cord has degenerated TH1+ axons and less numbers of motor neurons with no upregulation of D4a. The retinoic acid binding protein crabp2a is expressed in the pMN-like zone in the rostral spinal cord. The retinoic acid catabolizing enzyme, cyp26a is expressed in putative spinal cord progenitors at a higher level in the caudal than rostral spinal cord

List of Abbreviations

bHLH	basic-helix-loop-helix
BrdU	bromodeoxyuridine
Cdk	cyclin-dependent kinases
CGZ	circumferential germinal zone
CNTF	ciliary neurotrophic factor
CRABP2	cellular retinoic-acid-binding protein 2
Cven	commissural ventralis rhombencephali,
CYP26	Cytochrome P450 26
dpl	days post lesion
GCL	ganglion cell layer
GFAP	glial finrillary acidic protein
HD	homeodomain
her	hairy-related
Hh	hedgehog
HM	Hybridisation Mix
hpf	hours post fertilization
hs	heat-shocks
INL	inner nuclear layer
LIF	leukaemia inhibitory factor
mib	mind bomb

MS222	aminobenzoic acid ethylmethylester
NDS	normal donkey serum
NGS	normal goat serum
ONL	outer nuclear layer
PBS	phosphate buffered solution
PCNA	proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
RA	retinoic acid
RALDH	retinaldehyde dehydrogenases
RAR	RA receptor
RARE	retinoic acid-response element
RBP1	retinol-binding protein 1
RBP4	retinol-binding protein 4 plasma
RDH10	retinol dehydrogenase 10
RPE	retinal pigmented epithelial layer
RPE	retinal pigmented epithelium
RT	Reverse Transcriptase
RT	room temperature
RXR	retinoic X receptor
shh	sonic hedgehog

Sox	SRY-related HMG-box
TAE	Tris-acetate buffer
TTBc	tractus tectobulbaris cruciatus
VSX2	visual system homeobox 2
Wik	wildtype
wpl	weeks post lesion

List of Figures

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Scholarships and Awards

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2007-2010 College of Medicine and Veterinary Medicine PhD Studentship, University of Edinburgh (tuition: £10,200; bench fees: £3,000; living allowance: £40,030)

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2005-2006 College of Medicine and Veterinary Medicine scholarship for Masters by Research in Life Sciences, University of Edinburgh (£7,950)

Selected Conferences and Presentations

Oral presentations

07/2011 Scottish Developmental Biology Group Meeting, University of St.Andrews, UK

Title: The role of Notch signalling in the regenerating adult zebrafish spinal cord.

01/2011 Keystone Symposium on Adult Neurogenesis, New Mexico, U.S.A

Title: Spatial and temporal regulation of Delta-Notch signalling during spinal cord regeneration (selected speaker)

06/2008 Edinburgh Zebrafish Meeting, Edinburgh, UK

Title: Role of Notch signalling during spinal cord regeneration in adult zebrafish

Poster presentations

07/2011 7th European Zebrafish Meeting, EICC, Edinburgh UK
Title: Notch signaling controls generation of motor neurons in the lesioned spinal cord of adult zebrafish

01/2011 Keystone Symposium on Adult Neurogenesis, New Mexico, U.S.A.

Title: Spatial and temporal regulation of Delta-Notch signalling during spinal cord regeneration in adult zebrafish

12/2009 20th International Symposium on ALS/MND, Berlin, Germany

Title: Notch signaling in motor neuron regeneration in the lesioned spinal cord of adult zebrafish

Teaching experience

2010 – 2011 Demonstrator for Laboratory practicals & student evaluations, University of Edinburgh

Publication list

Submitted manuscripts

Dias TB, Yang YJ, Oogai K, Becker CG, Becker T. 2011. Notch signaling controls generation of motor neurons in the lesioned spinal cord of adult zebrafish. J Neurosci. Resubmitted 22 December 2011

Norris A, Kuscha V, Patani R, Reimer MM, Scott AL, Zhong Z, **Dias TB**, Frazer SL, Wyatt C, Chen YC, Ohnmacht J, Higashijima S, Patton EE, Panula P, Chandran S, Becker T, Becker CG. 2012. Dopamine/D4a signaling from the brain augments spinal motor neuron generation during development and adult regeneration via hedgehog pathway activation. in revision for re-submission

Referred manuscripts

Bridge KE, Berg N, Adalbert R, Babetto E, **Dias T**, Spillantini MG, Ribchester RR, Coleman MP. 2009. Late onset distal axonal swelling in YFP-H transgenic mice. Neurobiol Aging 30(2): 309-21

Published abstract

Dias T, Becker T, Becker C. Notch signaling in motor neuron regeneration in the lesioned spinal cord of adult zebrafish. 2009. Amyotrophic Lateral Sclerosis. Nov; 10 (1)